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DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

by

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SUMMARY

Groups of 15 male and 30 female (F_0 Parents) weanling rats were fed diets containing 0, 300, 1800 or 12000ppm DEHA. After 10 weeks, the animals were mated to produce a single litter (F_1A) which were reared to day 36 post partum. Test diets were fed continuously throughout the study.

There was no evidence for any clear effect on bodyweight or food consumption during the premating phase of the study. However, there was a reduction in bodyweight gain during gestation in the 12000ppm DEHA group compared with controls.

There were no treatment-related effects on pre-coital interval, length of gestation, or on male and female fertility.

Offspring weight gain, total litter weight and litter size in the 12000ppm DEHA group were reduced compared with controls, but there was no effect on the number of pups born live or on their survival at any level of DEHA.

An increase in liver weight was observed in both male and female parents receiving 12000ppm DEHA.

There were no histological changes in the reproductive organs of those males and females suspected of being infertile.

It is concluded that a dietary incorporation level of 12000ppm DEHA had no adverse effect on fertility in this study.

1. INTRODUCTION

Di-(2-ethylhexyl) adipate (DEHA) is a plasticiser for polyvinyl chloride particularly for low temperature application. The purpose of this study was to assess the effects of the continuous feeding of diets containing DEHA on the propagation of one generation of the Alpk:APfSD (Wistar-derived) strain of rats. The fertility of the parent animals and the clinical condition, survival and subsequent growth of their offspring was determined.

The Alpk:APfSD strain of rat was selected for this study as background clinical, reproductive and pathological data from similar studies relating to this strain are available in this Laboratory. The oral route of administration, with required levels of test substance added to the diet, was used as this is a possible route of human exposure and it is a route recommended by the OECD.

The dose levels for this study were based on data from the literature (NTP, 1982) and included an anticipated no effect level and a level at which toxic effects of DEHA were expected at some stage during the course of the study.

The study was conducted within the barriered Specific Pathogen Free (SPF) accommodation at ICI Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK. Experimental diets were first fed on 10 August 1987 and the last rats were sent for post mortem examination on 12 January 1988.

All original data pertaining to this study are retained in the Archives, ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK. Copies of the final report are kept in the Report Centre at CTL.

2. MATERIALS AND METHODS

2.1 Test Substance

DEHA was supplied by ICI France, Department Baleycourt, as a clear liquid with a purity of 99.2% w/w.

The CTL reference number assigned to this batch of DEHA was Y02259/003/001. A certificate of analysis is contained in Appendix A. The compound was stored in a ventilated cupboard in the CTL Central Dispensary at a temperature of approximately 20°C. Appropriate quantities of DEHA for incorporation into the diet were provided by the Central Dispensary at CTL, when required. A record of the dates of preparation and usage of diet was kept.

2.2 Diet

All diets were based on CT1 diet supplied by Special Diets Services (SDS) Ltd, Witham, Essex, UK, in 25kg sacks which were pasteurised on entry into the SPF Unit. The composition of CT1 diet is shown in Appendix B. The method of diet preparation is given in Appendix C.

Samples of the first batches of all dietary levels were taken from the food jars, prior to feeding, for quantitative analysis. Further samples were taken at regular intervals for full quantitative analysis.

The homogeneity and stability of DEHA in the CT1 diet was established on the first batch of diet prepared for the 300 and 12000ppm dose levels.

The analytical methods used for the determination of DEHA in the diet are shown in Appendices D and E. Vortex extraction of 2g diet samples (Appendix D) gave satisfactory data on fresh diet. However work carried out showed that low results may be obtained on stored diet and therefore Soxhlet extraction (Appendix E) was employed for many analyses, including those performed to measure chemical stability of DEHA in diet.

2.3 Animals

Alpk:APfSD (Wistar-derived) rats were obtained from the Specific Pathogen Free (SPF) colony maintained at the Alderley Park Breeding Unit, ICI, Alderley Park, Macclesfield, Cheshire, UK. All rats were supplied in single-sex litters as weanlings (approximately 21 days old) of known parentage.

They were transported to the barrier-maintained accommodation (SPF Unit, CTL) in sealed containers to maintain their SPF status. The rats arrived over a two day period, 3-4 August 1987. They were supplied as follows:

33 litters of at least 4 females
18 litters of at least 4 males

After delivery these rats were housed under SPF conditions, allowed to acclimatise to their environment for 6-7 days prior to the start of the study, and were provided with control CT1 diet ad libitum.

Filtered tap water was provided via an automatic watering system (North Kent Plastics Ltd, Dartford, Kent, UK). An additional supply was provided from water bottles fitted with ball-bearing nozzles (North Kent Plastics Ltd) for the first few days after arrival of the rats. This ensured that the rats obtained sufficient water until they were familiar with the automatic watering system.

For ten days following delivery of the rats from the Breeding Unit, personnel access to the animal room was restricted as a quarantine procedure. During this time the rats were observed once daily as a check on their health status.

2.4 Accommodation and Environment

On arrival the rats were housed by sex in litters. Following randomisation and during the premating period of the study they were housed, two females or one male per cage.

During pairing two females were housed with one male.

After mating all parent animals were individually housed.

Rats were housed in multiple rat racks supplied by All Type Tools (Woolwich) Ltd, London, UK. The cages had solid stainless steel sides; the floor, back and front were constructed of 14 standard wire gauge stainless steel mesh at 1.27cm centres. The cages were suspended over collecting trays lined with absorbent paper. A glass jar containing the appropriate diet was placed in each cage. Attached to each cage was a card uniquely identifying the animals in the cage.

The temperature in the animal rooms generally averaged a maximum of 21°C and minimum of 19°C (as recorded daily by a maximum and minimum thermometer) with occasional excursions outside this (overall range 16-24°C). There were fifteen to twenty-five air changes per hour. The daily relative humidity recorded generally averaged 45-60% (overall range 32-87%). The lighting was controlled by a time switch giving alternate periods of 12 hours light (07.00 to 19.00hrs) and 12 hours dark which were maintained throughout the study.

2.5 Experimental Design

After an acclimatisation period of 3-4 days the animals were distributed by litter amongst four groups to provide thirty females and fifteen males per group. Animals were started on study 3 days after randomisation. Details of parentage of each selected rat was documented. All animals were uniquely identified by ear punching. Rats not selected were discarded. The animal numbers are shown below.

TABLE 1
ANIMAL NUMBERS AND TREATMENT GROUPS

Group	Dietary Concentration of DEHA (ppm, w/w)	Animal Numbers	
		Males	Females
1	0	1 - 15	16 - 45
2	300	46 - 60	61 - 90
3	1800	91 - 105	106 - 135
4	12000	136 - 150	151 - 180

The groups were arranged on four racks in fifteen replicates using random permutations of the numbers 1-4, as shown in Appendix F. Each replicate consisted of two females and one male from each group.

Males and females from the same group were housed in adjacent cages during the period prior to mating to avoid anoestrous. The sequence of the groups within each replicate was determined by a shuffle card method (Appendix G). During gestation and lactation the females were housed individually and throughout this period the replicate design was maintained, with the males housed individually within their replicates but on a separate rack in another animal room. The second female replaced the male in the original cage in the replicate.

2.6 Parental Investigations

The F_0 parents were randomised by litter (Appendix G) before the start of the study. The procedure ensured that litter mates were evenly distributed across the groups. At randomisation, each rat was uniquely identified by ear punching with the number assigned to it by the experimental design. Treatment of the parents was started on 10 August 1987 and rats not required for the study were discarded. Details of the parentage of the F_0 rats were recorded.

The rats in each generation were fed experimental diets continuously until termination.

2.6.1 Clinical Condition: During the study all rats were observed daily for abnormalities in clinical condition and behaviour and once weekly a detailed examination of each rat was made. Any abnormalities were recorded as was the finding no abnormalities detected. Rats requiring euthanasia were sent for post mortem examination.

All females were examined prior to mating to detect any with imperforate vagina (a congenital abnormality of low incidence in the Alpk:APfSD rat), except for one female (number 76, 300ppm group) which was found to have

this condition after being placed with a male. This female was killed and subjected to a post mortem examination and was omitted from the reproductive performance parameters

2.6.2 Premating Period: The duration of the premating period was ten weeks from the start of the study.

(a) Bodyweights: Bodyweights of all rats were recorded at weekly intervals throughout the premating period. The initial weights for the F_0 parents were recorded immediately before first feeding experimental diets.

(b) Food Consumption and Food Utilisation: Food consumption for each cage of rats was recorded throughout the premating periods and calculated on a weekly basis, with the exception of several female cages where a set of residue and top up values were not recorded during the first week of the study. The food utilisation value per cage was calculated as the weight gained by the animals in the cage per 100g of food eaten.

2.6.3 Breeding Programme: Females were mated with a male of the same group and allowed to produce a litter.

During mating two females were housed with one male. Vaginal smears were examined daily to determine when mating occurred (as shown by the presence of sperm). If after ten days there was no evidence of mating the first male was removed and after at least three days was replaced by a second male from the same group of proven fertility. The rest period between the two pairing periods ensured that the paternity of any litter produced could be ascertained, should a positive vaginal smear have been missed. No further pairing was arranged if the second pairing was unsuccessful.

Any female with a positive vaginal smear was immediately separated from the male and individually housed. Pregnancy was presumed when abdominal enlargement and weight gain were seen. At approximately Day 15 of pregnancy the cages housing the females were fitted with solid floors and supplied with autoclaved paper bedding material. Water bottles were

also fitted to the cages at this time and the automatic watering system was disconnected. The females remained in these cages throughout gestation and lactation.

The female rats were weighed on Days 1, 8, 15 and 22 of pregnancy (the day on which sperm were detected in a vaginal smear was designated Day 1 of pregnancy), and at termination.

From the records of mating and parturition, the reproductive performance of the parents was assessed. The following were examined.

The fertility or otherwise of each male and female was established by the success of each mating. The criterion for successful mating was the production of a viable litter ie a litter in which at least one pup was found live at Day 1. The method of calculation is shown in Appendix H.

Length of gestation was measured in days from date of positive smear to date of birth (but only in fertile females fulfilling the criterion above).

Pre-coital interval, the time in days between the date of pairing and the date of positive smear, was measured, and mean value per group was estimated from all pairings with a positive smear.

In all pairings brother sister mating was avoided.

2.7 Offspring Investigations

2.7.1 Clinical Condition: Litters were examined for dead or moribund pups at least once daily and any such pups were subjected to a gross post mortem examination. For pups found dead up to and including 18 days of age, abnormalities were recorded and the pups were discarded. Pups over 18 days of age were examined as described in 2.8. A count of all live and dead pups was made within 24 hours of parturition (Day 1) and thereafter at Days 5, 11, 22, 29 and 36 post partum. The sexes of the pups were also recorded at these times. Any clinical abnormalities seen in the pups were recorded.

2.7.2 Bodyweights: Individual pup bodyweights were recorded within 24 hours of birth (Day 1) and at Days 5, 11, 22, 29 and 36 post partum. The litters were weaned at Day 22 post partum. Since pups were not individually identified, data were recorded by sex and litter.

2.8 Pathology

2.8.1 Procedures at Post Mortem Examination - Parents: All animals at scheduled kills and those killed during the study were anaesthetised by inhalation of halothane BP (FLUOTHANE, ICI Pharmaceuticals) vapour and killed by exsanguination.

All surviving male parents were killed after completion of mating. All female parents were killed after weaning their litters.

Any parent animal killed during the course of the study was subjected to the same post mortem procedure as animals surviving to termination.

All animals received a full necropsy in which the following tissues were submitted for possible histological examination:

Cervix	Prostate
Epididymis	Seminal vesicle
Liver	Testis
Mammary gland	Uterus
Ovary	Abnormal tissues

The number of implantation sites in each uterine horn was recorded for each mated female.

All tissues were submitted in 10% buffered formol saline except testis and epididymis and skin which were fixed in Bouin's solution and abnormal eyes which were fixed in Davidson's solution.

2.8.2 Procedures at Post Mortem Examination - Offspring: Moribund offspring and all offspring surviving to termination and those requiring euthanasia were killed as described for parents (Section 2.8.1) in the case of pups over 18 days, or by another method as appropriate in the case of younger pups.

Any pups (up to and including 18 days of age) found dead or with behavioural, functional, or morphological abnormalities were examined by free hand dissection.

Pups over 18 days of age found either dead or requiring euthanasia were subjected to a post mortem examination.

All pups were killed as soon as possible after Day 36 post partum.

All clinically abnormal pups and further normal pups received a gross necropsy so that a minimum of 2 pups of each sex were examined from each litter where possible. All remaining normal pups were killed and discarded after clinical examination.

2.8.3 Histology

Parents and Offspring

Histological examination of submitted tissues was as follows: Reproductive organs from F_0 animals which were suspected of infertility ie females failing to produce a viable litter and males failing to father any live pups. This definition was based on the records of live pups found at Day 1.

All other submitted tissues were stored.

2.9 Liver Weights

Liver weights were recorded from F_0 animals with the exception of those killed intercurrently.

2.10 Statistical Analyses

The following parameters were assessed using appropriate statistical tests: mean bodyweight gain, food consumption and food utilisation during the premating period, female bodyweight gain during pregnancy, parental liver weights and pup (litter) bodyweight gain until Day 36 post partum.

Male and female fertility indices, mean length of gestation, mean pre-coital interval, mean live born index, mean survival index, mean litter size, total litter weight and whole litter losses.

Female 125 (1800ppm DEHA) had a non-viable litter, hence only data pertaining to the premating period plus the organ weight, pre-coital interval and fertility data have been included in the analyses.

Details of the statistical procedures used are shown in Appendix I.

3. RESULTS

3.1 Diet Analysis (Table 2, Appendices J-L)

The majority of diets analysed had mean concentrations within 10% of target values (Appendix J). Only two diets at nominally 300ppm DEHA fell slightly outside these limits (110.7 and 112% of nominal concentration). DEHA was not detected in any control diet, detection limit 10ppm. Mean concentrations of all diets analysed were within 2% of target concentration for all dose groups (Table 2).

Chemical stability of DEHA in diet was determined on three batches of diet at nominally 300 and 12000ppm. Satisfactory chemical stability was established for diet stored at room temperature for up to 34 days (Appendix K).

Homogeneity of DEHA in diet mixtures was satisfactorily demonstrated on the first diet batch at nominally 300 and 12000ppm DEHA (Appendix L).

Diets were fed for no longer than three weeks from the date of preparation.

3.2 Parents

3.2.1 Clinical Condition and Mortality (Table 3): The rats in general remained in good clinical condition throughout the study, and there were no clinical abnormalities which could be attributed to treatment.

Two animals (1 male and 1 female), were killed because of their clinical condition. The incidence of these was unrelated to treatment.

3.2.2 Bodyweight and Bodyweight Gain - Premating Period (Table 4, Figures 1 and 2): Bodyweight gain was marginally reduced for females receiving 12000ppm DEHA. There was no effect on bodyweight gain in any other treatment group.

3.2.3 Food Consumption and Utilisation and Dose Rates - Premating Period (Tables 5-6, Figures 3 and 4, Appendix M): Food consumption for the F_0 females was based on weeks 2-10, and food utilisation from weeks 2-4, 5-7, 8-10 and 2-10.

There was a slight increase in food consumption in males dosed with 12000ppm DEHA from weeks 6-10 of the study, the effect being statistically significant at weeks 6-9. Food utilisation was slightly less efficient overall for males receiving 12000ppm DEHA. Food consumption and utilisation were unaffected for males receiving 300ppm or 1800ppm DEHA and females at all dose levels, when compared with control animals.

Dose rates (based on nominal dietary levels of DEHA) were calculated in terms of mg DEHA/kg bodyweight. As expected these were at a maximum in the early part of the study and declined during the phase of rapid growth (Appendix M).

3.2.4 Bodyweight and Bodyweight Gain - During Pregnancy (Table 7, Figure 2): The bodyweights of females treated with 12000ppm DEHA were marginally lower than controls at the start of pregnancy and there was reduced growth in this treatment group throughout pregnancy, the effect being most marked at weeks 3 and 4.

Day 22 mean bodyweights were approximately 6% lower in the 12000ppm treatment group than the control group. There was no effect on pregnancy bodyweight gain in females at the lower dose levels, when compared with control females.

3.2.5 Reproductive Performance (Tables 8, 9 and 10, Figures 5 and 6): There was no effect on either male or female fertility, gestation length or pre-coital interval in any dose group which could be attributed to treatment with DEHA.

3.3 Offspring (Tables 11-21, Figure 7)

The pups, in general, remained in good clinical condition. The incidence of clinical findings was low with no evidence of any relationship to treatment (Table 11).

There were four whole litter losses. None in the control group, one in the 300ppm DEHA dose group, two in the 1800ppm DEHA dose group and one in the 12000ppm DEHA dose group. These were of a low incidence and were not dose related. The incidence of whole litter losses was, therefore, not affected by treatment with DEHA (Table 12).

Mean pup weight gain and total litter weight for both male and female offspring receiving 12000ppm DEHA were reduced throughout the whole of the post partum phase ie Days 1-36. There was no effect on either male or female pup weight gain in any other dose group in comparison with the control animals (Tables 13-16).

Mean litter size was slightly reduced at the 12000ppm DEHA treatment level throughout the post partum period, no other treatment group was affected (Tables 17 and 18).

The number of live born pups was unaffected by treatment (Table 19) however, pup survival rate appeared to be marginally lower in all DEHA dose groups but when the whole litter losses were excluded there was no effect on pup survival at any treatment level (Tables 20 and 21).

Since the incidence of whole litter losses was not related to treatment, the apparent effect on pup survival was considered not to be related to treatment.

3.4 Liver Weights - Parents (Table 22)

Absolute liver weights were increased in both males and females receiving 12000ppm, by approximately 18%. A similar effect was observed when liver weight was adjusted for bodyweight; males receiving 12000ppm DEHA were increased by 18.9% and females by 19.7%. There was no effect on either absolute liver weight or liver weight adjusted for bodyweight in any other dose group.

3.5 Pathology

3.5.1 Parents

(a) Macroscopic Findings (Tables 23 and 24): There were no gross changes detected in adults which could be attributed to treatment with the possible exception of accentuated lobular pattern in the liver of two rats fed 12000ppm DEHA.

Implantation sites were absent from the uterus of most females which failed to litter but the number of affected animals was small and similar in control and treated groups.

(b) Microscopic Findings (Table 25): No microscopic changes were detected in the reproductive tract of animals which failed to breed successfully, to account for suspected infertility.

3.5.2 Offspring

(a) Macroscopic Findings (Table 26): There were no gross changes detected in the offspring which could be considered to be treatment related.

3.5.3 Visceral Examination of Offspring up to and including 18 Days of Age (Table 27): There were no macroscopic abnormalities in any of the pups subjected to gross necropsy which could be related to treatment with DEHA.

4. DISCUSSION

The study was completed according to schedule and there were no significant problems with environmental control of the accommodation or with study management. The health status of the rats was maintained at a high level and there was no evidence of disease or infection which might have compromised interpretation of the findings. The mating and production of the litter was completed satisfactorily.

Analysis of the diets for DEHA content established that only two diets were not within ±10% of the target concentrations and that the mean analysed concentrations were within ±2%.

No evidence for any clear effect on bodyweights or food consumption and utilisation was observed in any treatment group during the premating phase, apart from a marginal decrease in bodyweight gain in females receiving 12000ppm DEHA. This decrease in bodyweight gain continued through gestation and was statistically significant at weeks 3 and 4.

There was no evidence for any treatment-related effects on pre-coital interval, length of gestation, or on male and female fertility.

The offspring weight gain and total litter weight were reduced for male and female pups in the 12000ppm DEHA group. There was also a slight reduction in litter size throughout the post partum period in the 12000ppm treatment group. No effect on offspring weight gain, mean total litter weight or litter size was evident in offspring receiving 300ppm or 1800ppm DEHA. There was no effect on the number of pups born live or on pup survival at any level of DEHA.

An increase in liver weight was observed for both male and female parents receiving 12000ppm DEHA. No other treatment group was affected. This increase in liver weight has been reported previously and is associated with peroxisome proliferation (Moody and Reddy 1978).

There were no gross or microscopic pathological findings detected in either parents or offspring which could be attributed to treatment with DEHA with the possible exception of accentuated lobular pattern in two female parents fed 12000ppm DEHA. In particular, there were no histological changes in the reproductive organs of males or females suspected of being infertile.

5. CONCLUSION

The administration of 12000ppm DEHA in diet led to a reduction in female parent bodyweight gain during pregnancy and a reduction in offspring weight gain and litter size. No other reproductive parameters were affected and, in particular, there were no effects on fertility in either sex. Increased liver weights were also seen at 12000ppm in male and female parents.

It is concluded that a dietary incorporation level of 12000ppm DEHA had no adverse effect on fertility in this study.

6. REFERENCES

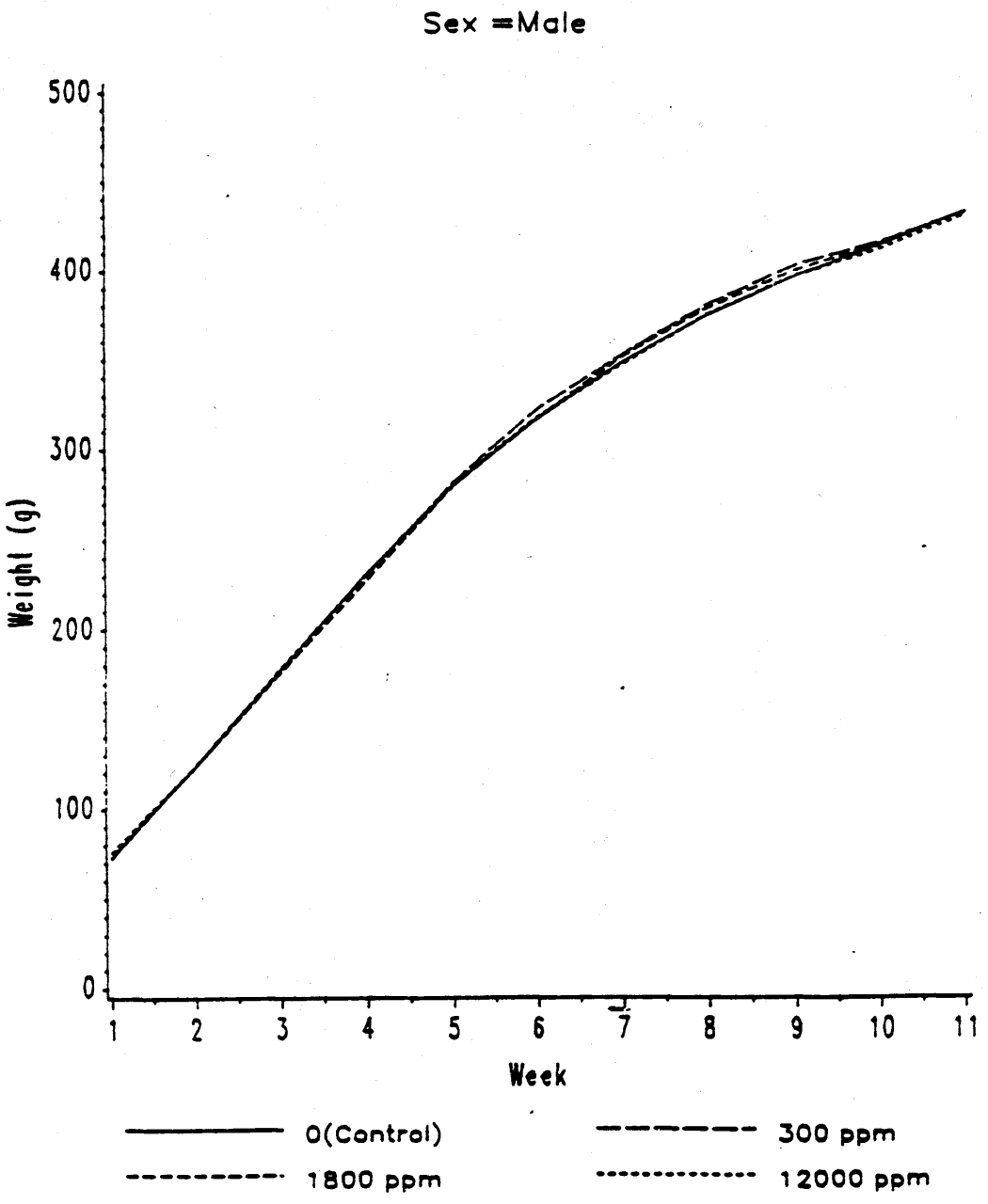
Moody D E, Reddy J K (1978). Hepatic peroxisome (microbody) proliferation in rats fed plasticizers and related compounds. Toxicol Appl Pharmacol 45 (2) 497-504.

National Toxicology Program Cancer Institute US DHHS (1982a). NTP Technical Report on the carcinogenesis bioassay of di(2-ethylhexyl)adipate (CAS No. 103-23-1). NTP Tech Rep Series No. 212 May.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

FIGURE 1

BODYWEIGHTS DURING PREMATING PERIOD - F_0 GENERATION

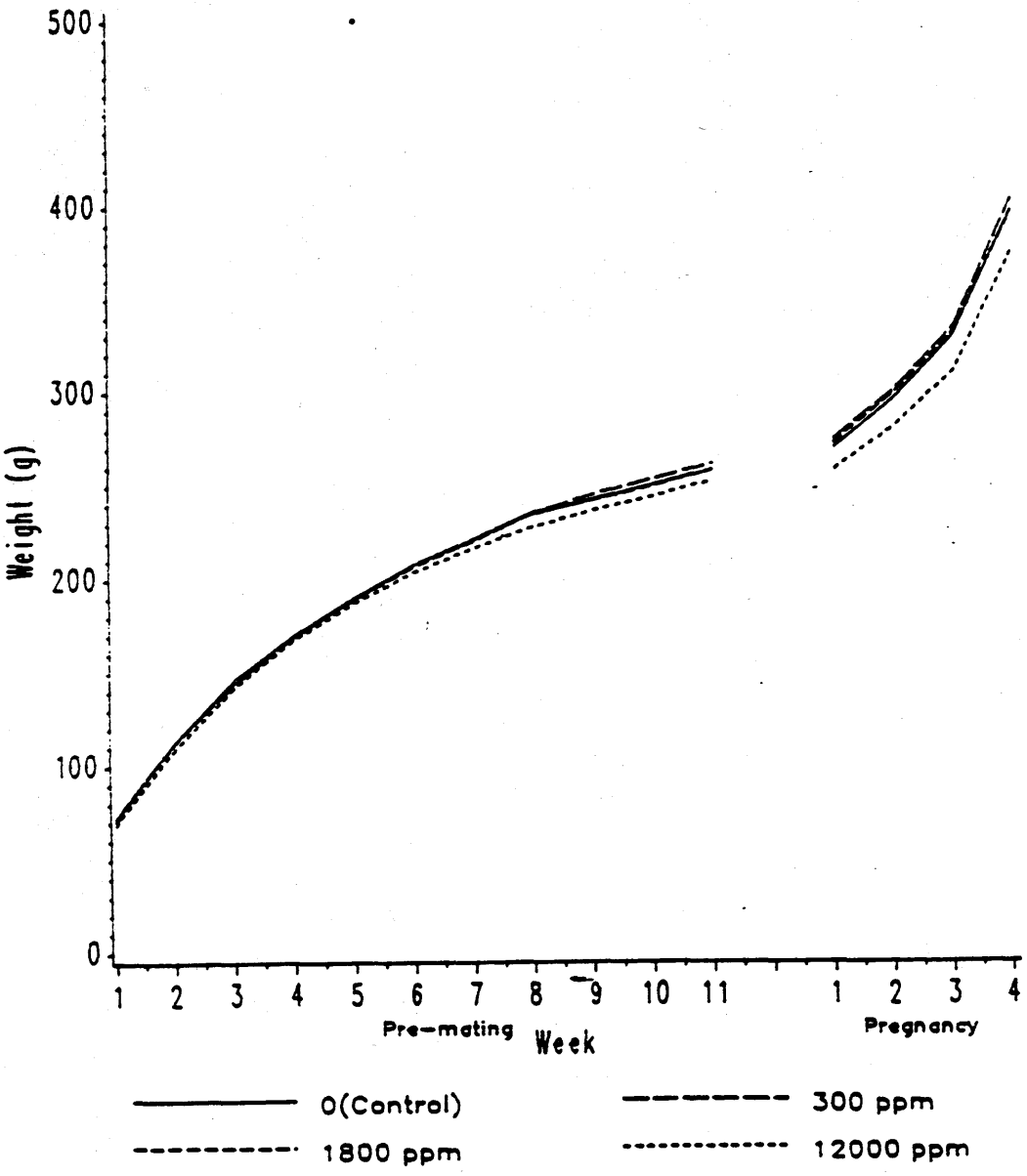


DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

FIGURE 2

BODYWEIGHTS DURING PREMATING PERIOD AND GESTATION PERIOD

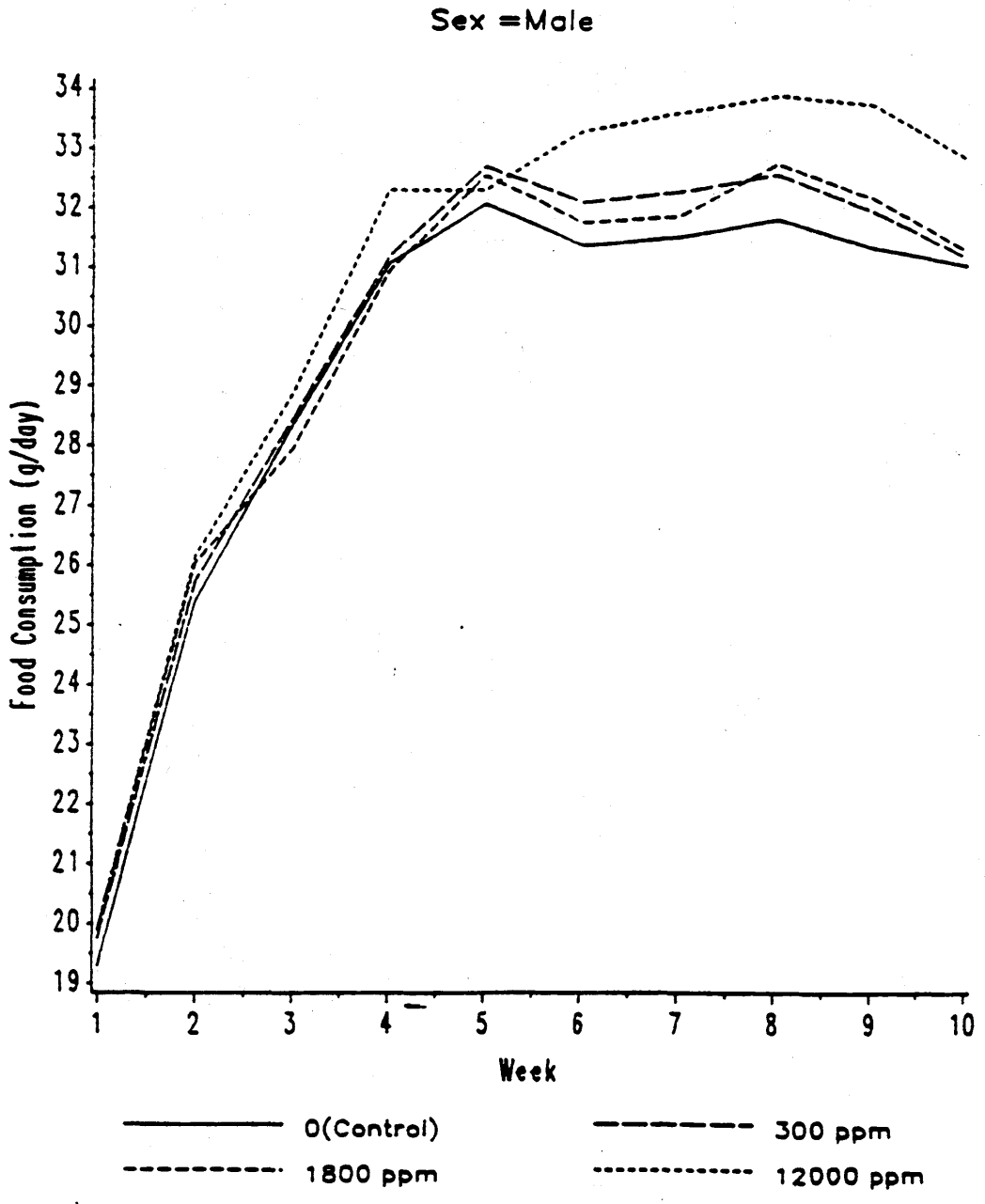
Sex - Female



DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

FIGURE 3

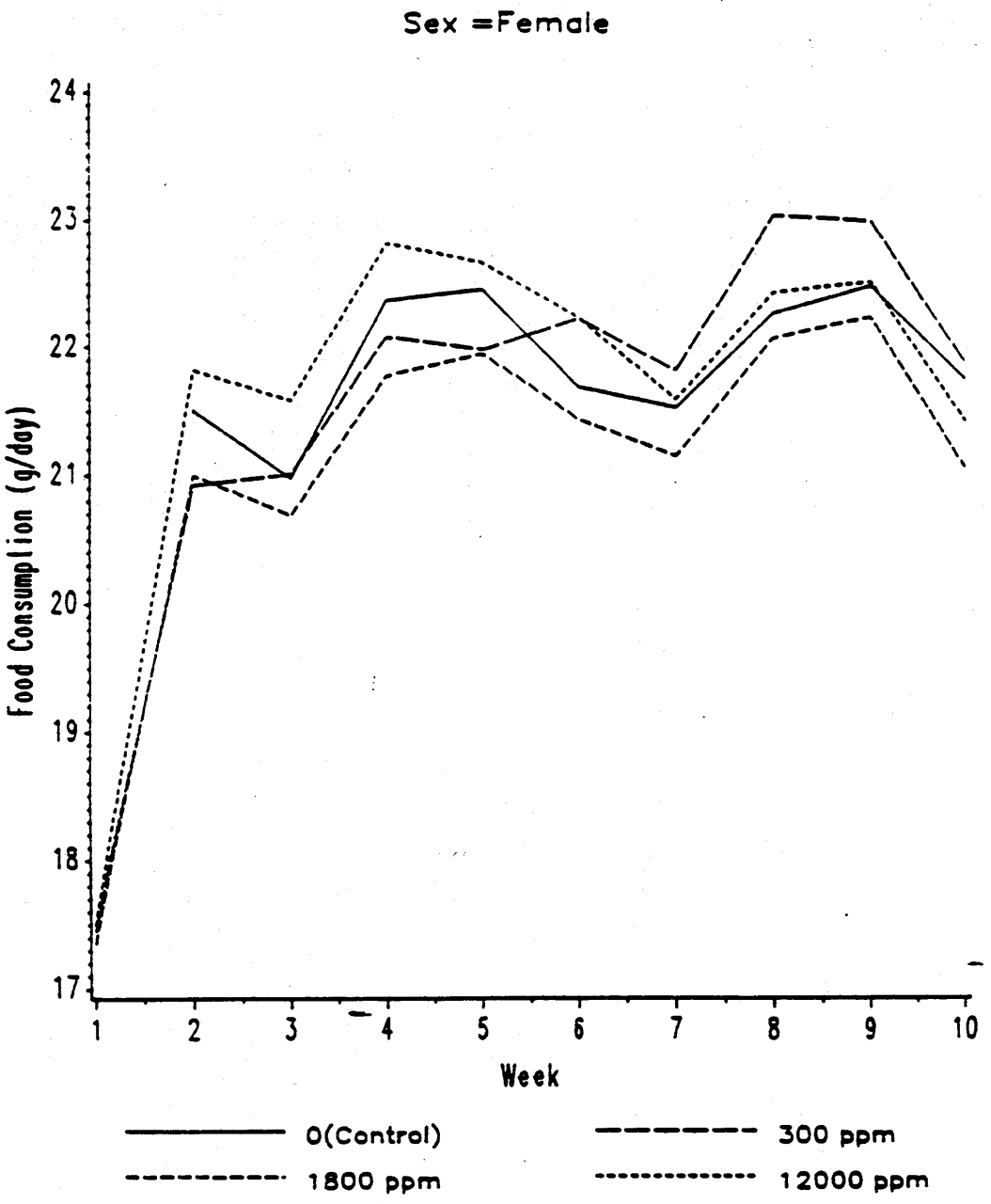
FOOD CONSUMPTION DURING PREMATING PERIOD - F_0 GENERATION



DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

FIGURE 4

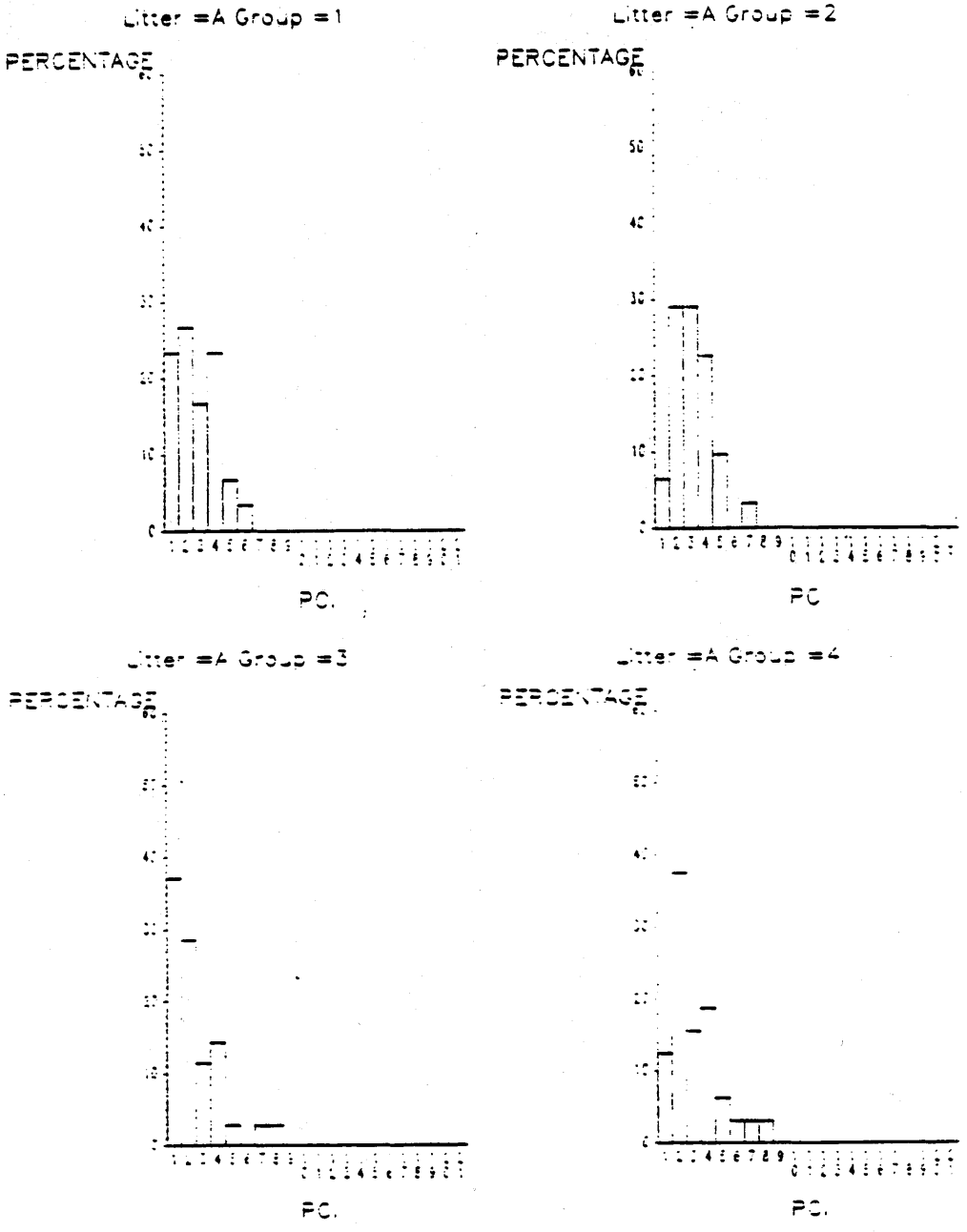
FOOD CONSUMPTION DURING PREMATING PERIOD - F_0 GENERATION



DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

FIGURE 5

PRE-COITAL INTERVAL (PCI-DAYS) F_0 PARENTS

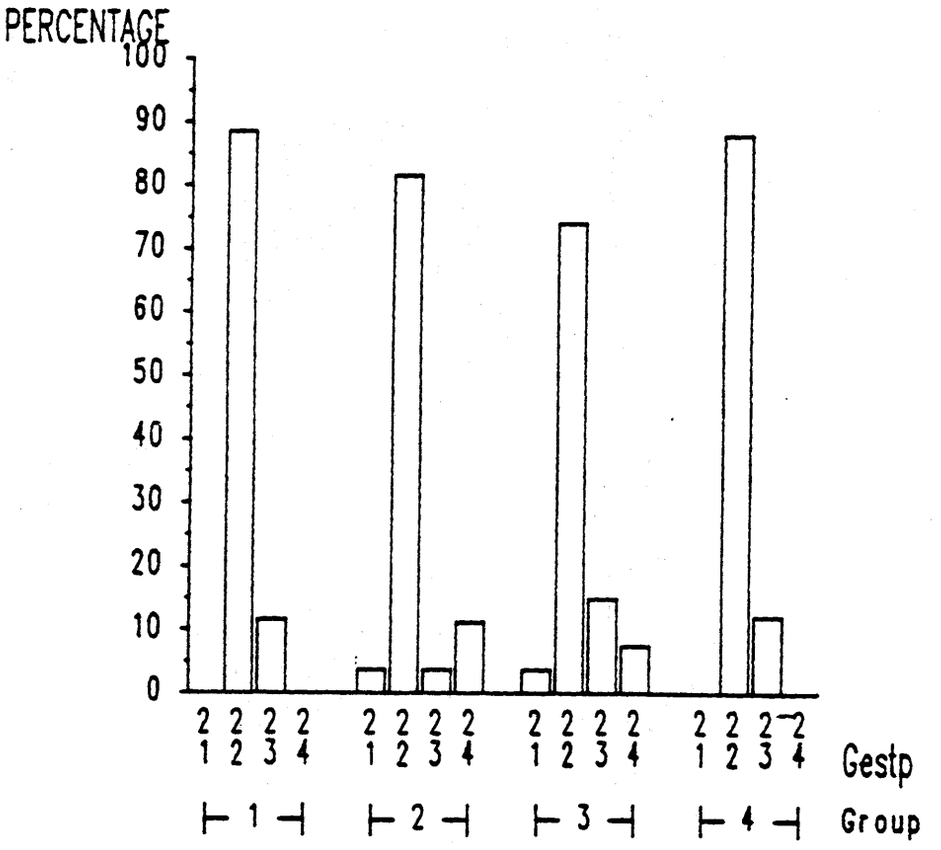


DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

FIGURE 6

GESTATION PERIOD (GESTP-DAYS) F_0 PARENTS

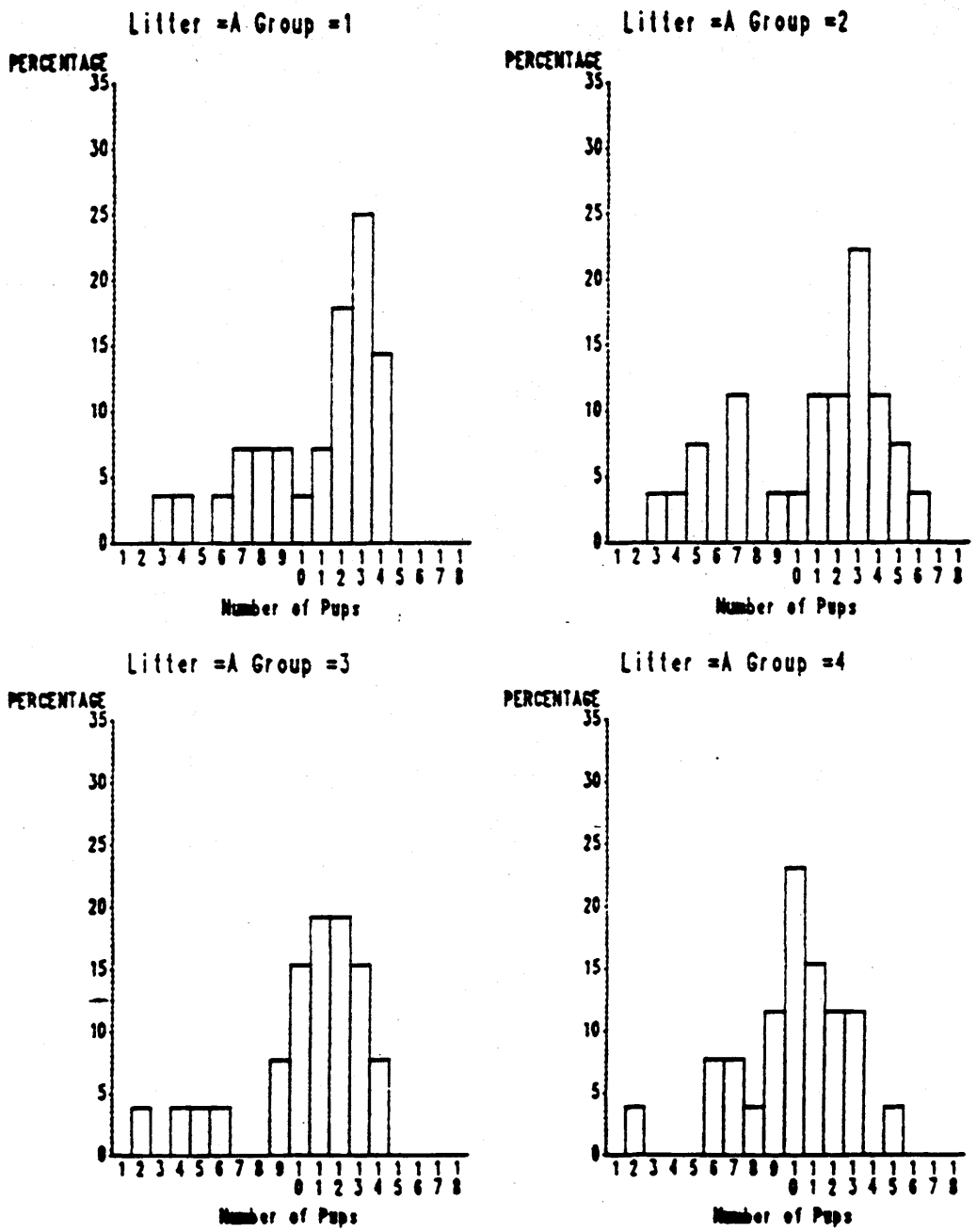
LITTER A



DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

FIGURE 7

LITTER SIZE - F_1 OFFSPRING - DAY 1 - POST PARTUM



Excludes whole litter losses.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

GLOSSARY FOR STATISTICAL TABLES

Means for parental bodyweight gain, food consumption and food utilisation are based on the following number of observations per group, unless otherwise indicated in brackets:

Bodyweight Gain	Males	-	15 observations
	Females	-	30 observations
Food Consumption		-	15 observations
Food Utilisation		-	15 observations

Means for all other tables are based on the number of observations in brackets.

Where confidence limits are presented means are least square means.

The approximate 95% confidence limit for each group mean is based on the error mean square in the analysis of variance or covariace and is calculated as the average 95% confidence limit for each individual group mean.

Key to results of statistical tests:

** Statistically significant difference from the control group mean at the 1% level (analysis as detailed in statistical methods section)

* Statistically significant difference from the control group mean at the 5% level (analysis as detailed in statistical methods section)

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

TABLE 2

SUMMARY OF DIET ANALYSIS

Nominal Concentration (ppm w/w)	Number of Diets Analysed	Mean Concentration (ppm w/w)	Concentration Range (ppm w/w)
300	7	304	287 - 336
1800	8	1830	1727 - 1954
12000	7	11840	10920 - 12610

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILTY STUDY IN RATS.

TABLE 3

INTERGROUP COMPARISON OF CLINICAL ABNORMALITIES - FO PARENTS

SEX: MALE	0 ppm		300 ppm		1800 ppm		12000 ppm	
DIARRHOEA								
NO. OF OBS.							1	
NO. OF ANIMALS							1	
WEEKS FROM - TO							6	6
KILLED IN EXTREMIS								
NO. OF OBS.							1	
NO. OF ANIMALS							1	
WEEKS FROM - TO							5	5
KILLED TERMINATION								
NO. OF OBS.	15		15		15		14	
NO. OF ANIMALS	15		15		15		14	
WEEKS FROM - TO	18	18	18	18	18	18	18	18
PILOERECTION								
NO. OF OBS.							1	
NO. OF ANIMALS							1	
WEEKS FROM - TO							5	5
CHROMODACRYORRHEA RIGHT								
NO. OF OBS.			9					
NO. OF ANIMALS			1					
WEEKS FROM - TO			3	11				
SCABS 1 OR MORE AREAS								
NO. OF OBS.	2							
NO. OF ANIMALS	1							
WEEKS FROM - TO	9	10						

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILTY STUDY IN RATS.

TABLE 3 - continued

INTERGROUP COMPARISON OF CLINICAL ABNORMALITIES - FO PARENTS

SEX: MALE	0 ppm	300 ppm	1800 ppm	12000 ppm
TAIL DAMAGED				
NO. OF OBS.			5	
NO. OF ANIMALS			1	
WEEKS FROM - TO			7 11	
THIN				
NO. OF OBS.				1
NO. OF ANIMALS				1
WEEKS FROM - TO				5 5

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILTY STUDY IN RATS.

TABLE 3 - continued

INTERGROUP COMPARISON OF CLINICAL ABNORMALITIES - FO PARENTS

SEX: FEMALE	0 ppm		300 ppm		1800 ppm		12000 ppm	
HAIR LOSS DORSALLY								
NO. OF OBS.			5					
NO. OF ANIMALS			1					
WEEKS FROM - TO			10	13				
HAIR LOSS 1 OR MORE AREAS								
NO. OF OBS.	2		1		1		2	
NO. OF ANIMALS	1		1		1		1	
WEEKS FROM - TO	6	6	3	3	13	13	18	18
KILLED:NO VAGINAL OPENING								
NO. OF OBS.			1					
NO. OF ANIMALS			1					
WEEKS FROM - TO			11	11				
KILLED TERMINATION								
NO. OF OBS.	30		29		30		30	
NO. OF ANIMALS	30		29		30		30	
WEEKS FROM - TO	18	23	18	23	18	23	18	23
MALOCCLUSION								
NO. OF OBS.			15					
NO. OF ANIMALS			1					
WEEKS FROM - TO			6	19				

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILTY STUDY IN RATS.

TABLE 3 - continued

INTERGROUP COMPARISON OF CLINICAL ABNORMALITIES - FO PARENTS

SEX: FEMALE	0 ppm	300 ppm	1800 ppm	12000 ppm
PILOERECTION				
NO. OF OBS.		1		
NO. OF ANIMALS		1		
WEEKS FROM - TO		6 6		
CHROMODACRYORRHEA RIGHT				
NO. OF OBS.		15	14	
NO. OF ANIMALS		1	1	
WEEKS FROM - TO		1 14	3 18	
SCABS 1 OR MORE AREAS				
NO. OF OBS.			1	
NO. OF ANIMALS			1	
WEEKS FROM - TO			11 11	
SUBCUTANEOUS MASS AREA 03				
NO. OF OBS.				1
NO. OF ANIMALS				1
WEEKS FROM - TO				18 18
SIGNS OF URINARY INCONTIN				
NO. OF OBS.	1			1
NO. OF ANIMALS	1			1
WEEKS FROM - TO	18 18			18 18
TAIL DAMAGED				
NO. OF OBS.		1		7
NO. OF ANIMALS		1		1
WEEKS FROM - TO		23 23		10 18

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILTY STUDY IN RATS.

TABLE 3 - continued

INTERGROUP COMPARISON OF CLINICAL ABNORMALITIES - FO PARENTS

SEX: FEMALE	0 ppm	300 ppm	1800 ppm	12000 ppm
TEETH TRIMMED				
NO. OF OBS.		12		
NO. OF ANIMALS		1		
WEEKS FROM - TO		6 18		
VAGINAL BLEEDING				
NO. OF OBS.	1			
NO. OF ANIMALS	1			
WEEKS FROM - TO	13 13			

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 4

INTERGROUP COMPARISON OF BODYWEIGHT GAIN (g)

F0 PARENTS

Period (Weeks)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
Males					
Initial Weight	72.5	73.0	73.4	75.8	-
1	50.7	51.0	49.9	48.1	±2.1
2	104.7	104.6	102.4	102.5	±4.0
3	158.6	157.0	153.3	154.2	±5.0
4	207.0	208.2	205.9	203.8	±6.2
5	244.5	249.2	243.4	241.2 (14)	±7.0
6	276.1	279.7	278.3	271.0 (14)	±8.1
7	302.4	307.7	305.3	298.8 (14)	±9.0
8	323.5	328.9	325.6	319.5 (14)	±9.9
9	341.1	342.1	340.3	334.5 (14)	±10.0
10	360.0	358.9	358.8	353.7 (14)	±11.1
Final Weight	432.5	431.9	432.2	429.6 (14)	±11.5

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 4 - continued

INTERGROUP COMPARISON OF BODYWEIGHT GAIN (g)

F0 PARENTS

Period (Weeks)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
Females					
Initial Weight	71.1	72.6	71.5	69.6	-
1	41.9	40.7	40.4	40.0	±1.4
2	75.2	71.1M	73.3	73.3	±2.7
3	99.0	97.3	98.0	98.8	±3.6
4	119.1	117.4	117.6	118.7	±4.5
5	136.4	135.4	135.3	134.8	±4.7
6	149.9	148.9	149.1	148.3	±5.7
7	163.6	162.9	163.8	160.1	±6.1
8	171.8	172.7	170.5	169.2	±6.6
9	179.7	181.0	178.3	176.2	±6.6
10	187.6	189.1	187.1	184.5	±6.8
Final Weight	258.7	261.7	258.6	254.1	±7.0

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 5

INTERGROUP COMPARISON OF FOOD CONSUMPTION (g/RAT/DAY)

F0 PARENTS

Period (Weeks)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
Males					
1	19.3	19.8	19.9	19.9	±1.0
2	25.4	25.7	26.0	26.2	±1.2
3	28.3	28.4	27.9	28.9	±0.9
4	31.1	31.2	30.9	32.3	±1.1
5	32.1	32.7	32.5	32.3	±2.1
6	31.4	32.1	31.7	33.3* (14)	±1.1
7	31.5	32.3	31.9	33.6** (14)	±1.1
8	31.8	32.5	32.7	33.8* (14)	±1.1
9	31.3	31.9	32.1	33.7** (14)	±1.2
10	31.0	31.2	31.3	32.8 (14)	±1.3
Total (1-10)	2052.0	2084.3	2079.5	2161.2* (14)	±62.6

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 5 - continued

INTERGROUP COMPARISON OF FOOD CONSUMPTION (g/RAT/DAY)

F0 PARENTS

Period (Weeks)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
Females					
1	0.0 (0)	17.5 (9)	17.4 (9)	17.5 (5)	-
2	21.5	20.9	21.0	21.8	±0.6
3	21.0	21.0	20.7	21.6	±0.6
4	22.4	22.1	21.8	22.8	±0.8
5	22.4	22.0	21.9	22.7	±1.0
6	21.7	22.2	21.4	22.2	±0.8
7	21.5	21.8	21.1	21.6	±0.7
8	22.3	23.0	22.1	22.4	±0.7
9	22.5	23.0	22.2	22.5	±0.7
10	21.7	21.9	21.1	21.4	±0.6
Total (2-10)	1378.6	1385.0	1353.1	1392.9	±34.6

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 6

INTERGROUP COMPARISON OF FOOD UTILISATION (g GROWTH / 100g FOOD)

F0 PARENTS

Period (Weeks)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
Males					
1 - 4	28.44	28.36	28.10	27.20*	±0.68
5 - 8	13.17	13.34	13.32	12.28 (14)	±0.84
9 -10	8.40	6.79*	7.45	7.41 (14)	±1.04
Overall (1-10)	17.58	17.25	17.29	16.41** (14)	±0.54

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 6 - continued

INTERGROUP COMPARISON OF FOOD UTILISATION (g GROWTH / 100g FOOD)

F0 PARENTS

Period (Weeks)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
Females					
2 - 4	17.04	17.12	17.39	17.01	±0.66
5 - 7	9.61	9.80	10.23	8.92	±0.76
8 -10	5.15	5.48	5.10	5.28	±0.67
Overall (2-10)	10.55	10.70	10.84	10.39	±0.38

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 7

INTERGROUP COMPARISON OF BODYWEIGHT GAIN (g) DURING PREGNANCY

F0 PARENTS

Period (Days)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
Litter A					
Initial Weight (Day 1)	270.5 (26)	277.0 (25)	268.3 (27)	260.2 (25)	±8.1
8	24.6 (26)	26.5 (25)	26.5 (27)	21.2 (25)	±2.7
15	59.1 (26)	60.7 (25)	59.1 (27)	50.4** (25)	±3.8
22	127.4 (26)	131.1 (25)	123.3 (27)	114.8** (25)	±6.0
Final Weight (Day 22)	398.0 (26)	404.7 (27)	391.6 (27)	374.0** (25)	±10.6

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 8

INTERGROUP COMPARISON OF FERTILITY (PROPORTION OF FERTILE ANIMALS)

	Dietary Concentration of DEHA (ppm)							
	0(Control)		300		1800		12000	
Males								
F1A Litter	15/15	100%	14/15	93%	14/15	93%	14/14	100%
Females								
F1A Litter	28/30	93%	28/29	97%	28/30	93%	27/30	90%

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 9

INTERGROUP COMPARISON OF PRE-COITAL INTERVAL (days)

F0 PARENTS

	Dietary Concentration of DEHA (ppm)							
	0(Control)		300		1800		12000	
Litter A								
No. and % of females with pre-coital interval								
1	7	23%	2	6%	13	37%	4	13%
2	8	27%	9	29%	10	29%	12	38%
3	5	17%	9	29%	4	11%	5	16%
4	7	23%	7	23%	5	14%	6	19%
>4	3	10%	4	13%	3	9%	5	16%
Mean pre-coital interval	2.73 (30)		3.13 (31)		2.43 (35)		3.06 (32)	

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 10

INTERGROUP COMPARISON OF GESTATION LENGTH (days)

F0 PARENTS

	Dietary Concentration of DEHA (ppm)							
	0(Control)		300		1800		12000	
Litter A								
No. and % of females with gestation length								
<22	0	0%	1	4%	1	4%	0	0%
22	23	88%	22	81%	20	74%	22	88%
>22	3	12%	4	15%	6	22%	3	12%
Mean gestation length	22.1 (26)		22.2 (27)		22.3 (27)		22.1 (25)	

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

TABLE 11

CLINICAL OBSERVATIONS - F_1 OFFSPRING

Clinical Observations		Dietary Concentration of DEHA (ppm)			
		0(Control)	30	1800	12000
Found dead (Cannibalised)	No. of Observations No. of Litters Days: From/To		10 2 4-5		1 1 25
Found dead	No. of Observations No. of Litters Days: From/To	13 11 1-29	15 9 1-34	14 6 1-25	8 5 1-2
Missing, presumed dead	No. Observations No. of Litters Days: From/To	18 10 5-11	25 14 3-29	24 10 3-11	26 10 3-7
Tip toe gait	No. of Observations No. of Litters Days: From/To			1 1 36	
Chromodacryorrhea one/both eyes	No. of Observations No. of Litters Days: From/To	1 1 36	8 2 22-36	3 1 22-36	
Hind limb damaged	No. of Observations No. of Litters Days: From/To				2 2 1
Hind limb part missing	No. of Observations No. of Litters Days: From/To				1 1 1
Stains around nose	No. of Observations No. of Litters Days: From/To			1 1 36	
Tail damaged	No. of Observations No. of Litters Days: From/To				1 1 1
Killed sick	No. of Observations No. of Litters Days: From/To	3 1 24		1 1 25	1 1 23

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

TABLE 11 - continued

CLINICAL OBSERVATIONS - F_1 OFFSPRING

Clinical Observations		Dietary Concentration of DEHA (ppm)			
		0(Control)	30	1800	12000
Thin	No. of Observations	1	1	2	2
	No. of Litters	1	1	2	2
	Days: From/To	5	5	1-36	1-5
Tip of tail blackened	No. of Observations	11			
	No. of Litters	1			
	Days: From/To	5-11			
Growth appears to have been stunted	No. Observations	6	2		5
	No. of Litters	3	2		4
	Days: From/To	36	36		36
Bruising to upper half of body	No. of Observations	1			
	No. of Litters	1			
	Days: From/To	1			
White cyst on abdomen	No. of Observations		1		
	No. of Litters		1		
	Days: From/To		5		
Moderately bruised head	No. of Observations			1	
	No. of Litters			1	
	Days: From/To			1	

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 12

INTERGROUP COMPARISON OF THE INCIDENCE OF WHOLE LITTER LOSSES

	Dietary Concentration of DEHA (ppm)							
	0(Control)		300		1800		12000	
F1A Litter	0/28	0%	1/28	4%	2/28	7%	1/27	4%

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 13

INTERGROUP COMPARISON OF PUP WEIGHT GAIN (g) - including whole litter losses

Period (Days)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
F1A Litter					
Males					
Initial Weight (Day 1)	5.9 (28)	6.0 (28)	6.1 (27)	6.1 (27)	±0.3
5	3.5 (28)	3.1 (27)	3.6 (25)	2.7** (26)	±0.4
11	12.4 (28)	11.9 (27)	13.1 (25)	10.5** (26)	±0.9
22	33.2 (28)	33.7 (27)	35.6 (25)	29.3* (26)	±2.5
29	65.9 (28)	65.9 (27)	69.2 (25)	57.2** (26)	±4.5
36	115.2 (28)	113.6 (27)	118.2 (25)	100.9** (26)	±6.4
Final Weight (Day 36)	121.1 (28)	119.6 (27)	124.3 (25)	107.0** (26)	±6.6
Females					
Initial Weight (Day 1)	5.5 (28)	5.6 (27)	5.8 (26)	5.7 (27)	±0.2
5	3.2 (28)	3.1 (26)	3.5 (24)	2.6 (27)	±0.4
11	11.8 (28)	11.7 (26)	12.6 (24)	10.5* (26)	±0.9
22	31.7 (28)	32.4 (26)	34.2 (24)	28.7 (26)	±2.4
29	61.5 (28)	61.4 (26)	64.4 (24)	54.7* (26)	±3.9
36	103.0 (28)	100.5 (26)	104.5 (24)	93.0** (26)	±5.3
Final Weight (Day 36)	108.6 (28)	106.0 (26)	110.3 (24)	98.7** (26)	±5.4

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 14

INTERGROUP COMPARISON OF PUP WEIGHT GAIN (g) - excluding whole litter losses

Period (Days)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
F1A Litter					
Males					
Initial Weight (Day 1)	5.9 (28)	6.0 (27)	6.2 (25)	6.1 (26)	±0.3
5	3.5 (28)	3.1 (27)	3.6 (25)	2.7** (26)	±0.4
11	12.4 (28)	11.9 (27)	13.1 (25)	10.5** (26)	±0.9
22	33.2 (28)	33.7 (27)	35.6 (25)	29.3* (26)	±2.5
29	65.9 (28)	65.9 (27)	69.2 (25)	57.2** (26)	±4.5
36	115.2 (28)	113.6 (27)	118.2 (25)	100.9** (26)	±6.4
Final Weight (Day 36)	121.1 (28)	119.6 (27)	124.3 (25)	107.0** (26)	±6.6
Females					
Initial Weight (Day 1)	5.5 (28)	5.5 (26)	5.8 (25)	5.7 (26)	±0.2
5	3.2 (28)	3.1 (26)	3.5 (24)	2.7 (26)	±0.4
11	11.8 (28)	11.7 (26)	12.6 (24)	10.5* (26)	±0.9
22	31.7 (28)	32.4 (26)	34.2 (24)	28.7 (26)	±2.4
29	61.5 (28)	61.4 (26)	64.4 (24)	54.7* (26)	±3.9
36	103.0 (28)	100.5 (26)	104.5 (24)	93.0** (26)	±5.3
Final Weight (Day 36)	108.6 (28)	106.0 (26)	110.3 (24)	98.7** (26)	±5.4

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 15

INTERGROUP COMPARISON OF TOTAL LITTER WEIGHT (g) - including whole litter losses

Period (Days)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
F1A Litter					
Initial Weight	61.8 (28)	61.9 (28)	58.9 (28)	57.0 (27)	±6.0
Day 5	91.3 (28)	86.4 (27)	92.7 (26)	74.8* (27)	±9.9
Day 11	176.7 (28)	170.2 (27)	182.5 (26)	146.0* (26)	±17.2
Day 22	375.2 (28)	369.9 (27)	393.5 (26)	303.2** (26)	±33.7
Day 29	672.2 (28)	662.1 (27)	696.4 (26)	530.0** (26)	±65.1
Day 36	1118.2 (28)	1079.8 (27)	1128.5 (26)	892.8** (26)	±107.5

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 16

INTERGROUP COMPARISON OF TOTAL LITTER WEIGHT (g) - excluding whole litter losses

Period (Days)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
F1A Litter					
Initial Weight	61.7 (28)	61.8 (27)	60.6 (26)	57.6 (26)	±5.9
Day 5	91.3 (28)	86.3 (27)	92.6 (26)	77.5* (26)	±9.6
Day 11	176.7 (28)	170.2 (27)	182.5 (26)	146.0* (26)	±17.2
Day 22	375.2 (28)	369.9 (27)	393.5 (26)	303.2** (26)	±33.7
Day 29	672.2 (28)	662.1 (27)	696.4 (26)	530.0** (26)	±65.1
Day 36	1118.2 (28)	1079.8 (27)	1128.5 (26)	892.8** (26)	±107.5

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 17

INTERGROUP COMPARISON OF LITTER SIZE - Including whole litter losses

Period (Days)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
F1A Litter					
Day 1	10.9 (28)	10.8 (28)	10.1 (28)	9.7 (27)	±1.1
Day 5	10.2 (28)	9.6 (28)	9.3 (28)	8.6 (27)	±1.2
Day 11	10.2 (28)	9.5 (28)	9.3 (28)	8.6 (27)	±1.2
Day 22	10.2 (28)	9.5 (28)	9.3 (28)	8.6 (27)	±1.2
Day 29	10.0 (28)	9.5 (28)	9.1 (28)	8.5 (27)	±1.2
Day 36	10.0 (28)	9.4 (28)	9.1 (28)	8.5 (27)	±1.2

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 18

INTERGROUP COMPARISON OF LITTER SIZE - excluding whole litter losses

Period (Days)	Dietary Concentration of DEHA (ppm) 0(Control)	300	1800	12000	Approx 95% Conf Limit
F1A Litter					
Day 1	10.9 (28)	10.8 (27)	10.3 (26)	9.8 (26)	±1.2
Day 5	10.2 (28)	9.8 (27)	10.0 (26)	8.9 (26)	±1.1
Day 11	10.1 (28)	9.8 (27)	9.9 (26)	8.9 (26)	±1.1
Day 22	10.1 (28)	9.8 (27)	9.9 (26)	8.9 (26)	±1.1
Day 29	9.9 (28)	9.7 (27)	9.7 (26)	8.8 (26)	±1.1
Day 36	9.9 (28)	9.7 (27)	9.7 (26)	8.8 (26)	±1.1

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 19

INTERGROUP COMPARISON OF PUPS LIVE BORN - including Whole Litter Losses

	Dietary Concentration of DEHA (ppm)				Approx 95% Conf Limit
	0(Control)	300	1800	12000	
F1A Litter					
Mean percentage	97.5	95.8	98.8	99.3	-
Prop. of litters with all pups live born	21/28	21/28	24/28	25/27	

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 20

INTERGROUP COMPARISON OF PUPS SURVIVING TO DAY 22 - including Whole Litter Losses

	Dietary Concentration of DEHA (ppm)				
	0(Control)	300	1800	12000	Approx 95% Conf Limit
F1A Litter					
Mean percentage	94.1	88.7	89.3	87.6	-
Prop. of litters with all pups surviving to day 22	18/28	12/28	18/28	16/27	

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 21

INTERGROUP COMPARISON OF PUPS SURVIVING TO DAY 22 - excluding Whole Litter Losses

	Dietary Concentration of DEHA (ppm)				Approx 95%
	0(Control)	300	1800	12000	Conf Limit
F1A Litter					
Mean percentage	94.1	92.0	96.2	91.0	-
Prop. of litters with all pups surviving to day 22	18/28	12/27	18/26	16/26	

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 22

INTERGROUP COMPARISON OF ORGAN WEIGHTS

F0 PARENTS

	Dietary Concentration of DEHA (ppm)				
	0(Control)	300	1800	12000	Approx 95% Conf Limit
Liver					
Males					
Organ Weight (g)	19.1 (15)	19.7 (15)	19.5 (15)	22.5** (14)	±1.0
Adjusted For Bodyweight (g)	19.0 (15)	19.7 (15)	19.5 (15)	22.6** (14)	±0.8
Females					
Organ Weight (g)	13.2 (30)	13.2 (29)	13.3 (30)	15.5** (30)	±0.7
Adjusted For Bodyweight (g)	13.2 (30)	13.0 (29)	13.2 (30)	15.8** (30)	±0.6

DI-(2- ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 23

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS - F0 PARENTS

SEX: MALES	DIETARY CONCENTRATION OF DEHA 0 ppm	300 ppm	1800 ppm	12000 ppm
MALES ON STUDY	15	15	15	15
ANIMALS COMPLETED	15	15	15	15
BLADDER				
SUBMITTED	0	2	0	0
NO.WITH FINDINGS	0	2	0	0
White deposit	0	2	0	0
EPIDIDYMIS				
SUBMITTED	15	15	15	15
NO.WITH FINDINGS	0	0	0	1
NO.WITHOUT FINDINGS	15	15	15	14
Enlarged	0	0	0	1
KIDNEY				
SUBMITTED	1	0	1	0
NO.WITH FINDINGS	1	0	1	0
Pelvic dilatation	1	0	1	0
LIVER				
SUBMITTED	15	15	15	15
NO.WITH FINDINGS	0	0	0	1
NO.WITHOUT FINDINGS	15	15	15	14
Dark	0	0	0	1
SEMINAL VESICLE				
SUBMITTED	15	15	15	15
NO.WITH FINDINGS	1	1	0	1
NO.WITHOUT FINDINGS	14	14	15	14
Reduced	1	1	0	1

DI-(2- ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 23 - continued
INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS - FO PARENTS

SEX: FEMALES	DIETARY CONCENTRATION OF DEHA 0 ppm	300 ppm	1800 ppm	12000 ppm
FEMALES ON STUDY	30	30	30	30
ANIMALS COMPLETED	30	30	30	30
BLADDER				
NO.WITH FINDINGS BUT NOT SUBMITTED	0	0	1	0
White deposit	0	0	1	0
Walls thickened	0	0	1	0
DIAPHRAGM				
SUBMITTED	1	0	0	0
NO.WITH FINDINGS	1	0	0	0
Hernia	1	0	0	0
EYE				
SUBMITTED	0	0	1	0
NO.WITH FINDINGS	0	0	1	0
Chromodacryorrhea	0	0	1	0
KIDNEY				
SUBMITTED	1	3	2	1
NO.WITH FINDINGS	1	3	2	1
Pelvic dilatation	1	3	2	1
Deposit/s pelvis	0	0	0	1
LIVER				
SUBMITTED	30	30	30	30
NO.WITH FINDINGS	1	0	0	3
NO.WITHOUT FINDINGS	29	30	30	27
Accentuated lobular pattern	0	0	0	2
Enlarged	0	0	0	1
Raised area	1	0	0	0
MAMMARY GLAND				
SUBMITTED	30	30	30	30
NO.WITH FINDINGS	1	0	1	1
NO.WITHOUT FINDINGS	29	30	29	29
Firm area/s	0	0	0	1
Red	1	0	0	0
Nipple scabs	0	0	1	0

DI-(2- ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 23 - continued
INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS - F0 PARENTS

SEX: FEMALES	DIETARY CONCENTRATION OF DEHA 0 ppm	300 ppm	1800 ppm	12000 ppm
FEMALES ON STUDY	30	30	30	30
ANIMALS COMPLETED	30	30	30	30
OVARY				
SUBMITTED	30	30	30	30
NO.WITH FINDINGS	1	2	4	1
NO.WITHOUT FINDINGS	29	28	26	29
Cystic bursa	1	2	4	0
Cystic	0	0	0	1
SKIN				
SUBMITTED	0	0	0	1
NO.WITH FINDINGS	0	0	0	1
Hair loss	0	0	0	1
STOMACH				
SUBMITTED	0	0	0	1
NO.WITH FINDINGS	0	0	0	1
Abnormal contents	0	0	0	1
UTERUS				
SUBMITTED	30	30	30	30
NO.WITH FINDINGS	30	29	30	30
NO.WITHOUT FINDINGS	0	1	0	0
Distended	0	3	0	1
Implantation sites present	28	29	29	28
Implantation sites absent	2	0	1	2
Blood filled	0	0	0	1
VAGINA				
SUBMITTED	0	1	0	0
NO.WITH FINDINGS	0	1	0	0
Enlarged	0	1	0	0
Imperforate	0	1	0	0

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 24

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS IN THE REPRODUCTIVE TRACT OF FO MALES: A COMPARISON BETWEEN ANIMALS WITH NORMAL AND ABNORMAL BREEDING RECORDS

Tissue/Pathological Findings	Normal Breeders				Abnormal Breeders			
	Dose (ppm DEHA)							
	0	300	1800	12000	0	300	1800	12000
Number of animals examined:	15	14	14	15	0	1	1	0
Seminal vesicle								
Reduced	0	0	0	1*	-	-	-	-

* Includes number 140 - killed intercurrently after 33 days

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 24 (continued)

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS IN THE REPRODUCTIVE TRACT OF FO FEMALES:
A COMPARISON BETWEEN ANIMALS WITH NORMAL AND ABNORMAL BREEDING RECORDS

Tissue/Pathological Findings	Normal Breeders				Abnormal Breeders			
	Dose (ppm DEHA)							
	0	300	1800	12000	0	300	1800	12000
Number of animals examined:	28	28	28	27	2	2*	2	3
Uterus:								
Implantation sites absent	-	-	-	-	2	0	1	2
Implantation sites present	-	-	-	-	0	1	1	1
Vagina:								
Imperforate - distended with fluid	-	-	-	-	0	1*	0	0

* Includes Number 76 - killed intercurrently after 73 days

DI-(2- ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 25
INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS - FO PARENTS

SEX: MALES	DIETARY CONCENTRATION OF DEHA 0 ppm	300 ppm	1800 ppm	12000 ppm
MALES ON STUDY	15	15	15	15
ANIMALS COMPLETED	0	1	1	1
SEMINAL VESICLE				
EXAMINED	0	1	1	1
NO ABNORMALITIES DETECTED	0	1	1	0
Reduced quantity of secretion (TOTAL)	0	0	0	1
Moderate	0	0	0	1

DI-(2- ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 25
INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS - FO PARENTS

SEX: FEMALES	DIETARY CONCENTRATION OF DEHA 0 ppm	300 ppm	1800 ppm	12000 ppm
FEMALES ON STUDY	30	30	30	30
ANIMALS COMPLETED	2	2	2	3
CERVIX				
EXAMINED	2	2	2	3
NO ABNORMALITIES DETECTED	2	1	2	3
Distended (TOTAL)	0	1	0	0
Marked	0	1	0	0
UTERUS				
EXAMINED	2	2	2	3
NO ABNORMALITIES DETECTED	2	2	1	3
Glandular dilatation (TOTAL)	0	0	1	0
Minimal	0	0	1	0
VAGINA				
EXAMINED	0	1	0	0
Distended (TOTAL)	0	1	0	0
Marked	0	1	0	0
Imperforate (diagnosis based on macroscopic observations.)	0	1	0	0

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 26

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS IN F1 OFFSPRING*

Tissue/Pathological Findings	Males				Females			
	Dose (ppm DEHA)							
	0	300	1800	12000	0	300	1800	12000
Number of animals examined:	66(3)	54	53(3)	53	58(3)	56(2)	52(2)	49(1)
Bladder								
Distended	3(3)	0	0	0	0	1(1)	0	0
Thickened	0	0	0	0	0	1(1)	0	0
Buccal Cavity								
Malocclusion	0	0	0	0	0	1	0	0
Caecum								
Distended	0	0	0	0	0	1	0	0
Cervix								
Distended	-	-	-	-	0	1	0	0

* Figures in brackets indicate number of intercurrent deaths included in total for a given finding

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 26 (continued)

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS IN F1 OFFSPRING*

Tissue/Pathological Findings	Males				Females			
	Dose (ppm DEHA)							
	0	300	1800	12000	0	300	1800	12000
Number of animals examined:	66(3)	54	53(3)	53	58(3)	56(2)	52(2)	49(1)
Diaphragm								
Hernia	1	0	0	0	0	0	0	0
Eye								
Chromodacryorrhea	1	1	0	0	0	2	1	0
Ileum/Jejunum								
Prominent Peyers Patches	0	0	0	0	0	0	2	0

* Figures in brackets indicate number of intercurrent deaths included in total for a given finding

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 26 (continued)

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS IN F1 OFFSPRING*

Tissue/Pathological Findings	Males				Females			
	Dose (ppm DEHA)							
	0	300	1800	12000	0	300	1800	12000
Number of animals examined:	66(3)	54	53(3)	53	58(3)	56(2)	52(2)	49(1)
Kidney								
Cyst	1	0	0	0	0	0	0	0
Discolouration - focal	0	0	0	0	0	1(1)	0	0
Enlarged, flaccid, nodular	0	0	0	0	0	0	1	0
Pelvic dilatation - unilateral	7	8	10	7	3	4	2	1
- bilateral	1(1)	0	0	0	1	2(1)	1	0
Pelvis thickened, white	0	0	0	0	0	0	1	0
Soft deposit	0	0	0	0	0	0	1	0

* Figures in brackets indicate number of intercurrent deaths included in total for a given finding

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 26 (continued)

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS IN F1 OFFSPRING*

Tissue/Pathological Findings	Males				Females			
	Dose (ppm DEHA)							
	0	300	1800	12000	0	300	1800	12000
Number of animals examined:	66(3)	54	53(3)	53	58(3)	56(2)	52(2)	49(1)
Liver								
Adhesion	0	0	1	0	0	0	0	0
Cyst	0	0	0	0	0	0	1	0
Herniated portion	1	0	0	0	0	0	0	0
Pale	0	0	0	2	0	0	0	0
Reddened areas	0	0	0	1	0	0	0	0
Speckled	0	0	0	1	0	0	0	0
Lung								
Discolouration - focal	0	1	1	0	1	0	2	1

* Figures in brackets indicate number of intercurrent deaths included in total for a given finding

DI-(2-ETHYLHEXYL) ADIPATE (DEHA) : FERTILITY STUDY IN RATS

TABLE 26 (continued)

INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS IN F1 OFFSPRING*

Tissue/Pathological Findings	Males				Females			
	Dose (ppm DEHA)							
	0	300	1800	12000	0	300	1800	12000
Number of animals examined:	66(3)	54	53(3)	53	58(3)	56(2)	52(2)	49(1)
Skin								
Stained hair	0	0	1	0	0	0	0	0
Testis								
Reduced (unilateral)	1	1	0	0	-	-	-	-
Uterus								
Distended	0	0	0	0	0	2	0	0

* Figures in brackets indicate number of intercurrent deaths included in total for a given finding

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

TABLE 27

GROSS NECROPSY OBSERVATIONS IN PUPS FOUND DEAD UPTO AND INCLUDING 18 DAYS OF AGE

Litter	Observations	Dietary Concentration of DEHA (ppm)			
		0 (Control)	30	1800	12000
F_1	Number of pups examined	10	14	10	8
	No abnormalities detected	1	2	-	-
	Abdominal organs autolysed - otherwise no abnormalities diagnosed	8	7	4 & 2[B]	1
	Abdominal and thoracic organs autolysed - otherwise no abnormalities diagnosed	-	1[A]	-	3[C]
	Complete autolysis	-	1	-	-
	Bladder distended	-	2	-	-
	Milk present in stomach - otherwise no abnormalities diagnosed	-	1	1	3
	Yellow gaseous fluid in stomach - otherwise no abnormalities diagnosed	1	-	3	-
	Clear gaseous fluid in stomach - otherwise no abnormalities diagnosed	-	-	-	1

A = Unable to sex.
B = Also, forelimbs, hindlimbs and tail autolysed.
C = Stomach and intestines gas filled.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX A

CERTIFICATE OF ANALYSIS

Y02259/003/001

ZCZC NPA742 MAB027 10 1548
QN PHNAICI
.MOHQICI

10/6/87 MOAES ST1134

TO: P WILLIAMS CENTRAL TOXICOLOGICAL LABS ALDERLY PARK

HEXAPLAS DOA SANS ODEUR: 2 X 5 L

ANALYTICAL CERTIFICATE:

- ESTER CONTENT (GLC) AT W/W DOA	99.2
- PHTHALATE CONTENT (UV SPECTROPHOTOMETER) AS DOP AT W/W	0.08
- FREE ALCOHOL CONTENT (AT W/W)	0.02
- WATER CONTENT (AT W/W)	0.04
- ACID VALUE (MG KOH/G)	0.017

THESE TWO SAMPLES ARE SENT TOGETHER TODAY IN ONE WOODEN BOX, AND DELIVERED ON THE 11/06/87 DIRECTLY TO DR. G. STEEL BY STE TNT - IPEC.

REGARDS.

FROM: EVC R+TS RUNCORN HEATH RES BLK
D F CADOGAN

NNNN

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX B

CT1 DIET

Supplied by Special Diets Services Limited, Stepfield, Witham, Essex.

CT1 diet was supplied as a meal in 25kg quantities which were wrapped in 5 ply paper sacks. An analysis of each batch of diet for major constituents and contaminants was supplied by Special Diets Services Limited. This was checked for acceptability (based on the best available information at the time) before the batch was used on the study.

The known contaminants found in the diet were not considered to be present in sufficient concentration to have an influence on the outcome of the study.

CT1 diet was prepared from the following fixed formula:

	% w/w
Cornflour	10.0
Wheat Bran	15.0
Wheat	20.0
Maize	10.0
Wheat Feed	20.0
Soya Hypro 50	8.0
Unextracted Yeast	2.5
Denatured Skim Milk Powder*	7.5
White Fish Meal	5.0
PCD Premix	2.0

* Denatured skim milk powder has the following formula:

Skim Milk Powder	72%
White Fish Meal	28%

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX B - continued

CT1 DIET

When used at 2% inclusion rate (20kg/tonne) PCD premix contributes the following.

Vitamin A	8.0 m.i.u.
Vitamin D_3	1.0 m.i.u
Vitamin E	62.5g
Vitamin B_2	8.0g
Vitamin K M.S.B.	10.0g
Nicotinic Acid	20.0g
Pantothenic Acid	4.4g
Folic Acid	6.0g
Vitamin B_1	2.0g
Vitamin B_{12}	12.0g
Choline	150.0g
Iron	30.0g
Cobalt	0.4g
Manganese	25.0g
Copper	7.0g
Iodine	1.3g
Magnesium	103.0g
Sodium Chloride	5000.0g
Phosphorus	1200.0g
Calcium	4480.0g

All batches of CT1 diet comply with the following specification with respect to the maximum permitted levels of contaminants.

Contaminant	Maximum permitted level (ppm)	
Selenium	(min)	0.025
Selenium	(max)	0.5
Cadmium		0.8
Mercury		0.2
Arsenic		1.0
Lead		3.0
PCB's	(total)	0.15
DDT's	(total)	0.3
Dieldrin		0.05
Lindane		0.1
Heptachlor		0.05
Malathion		5.0
Nitrite		5.0
Nitrate		150.0
Aflatoxin		0.01

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX C

PREPARATION OF EXPERIMENTAL DIETS

Diets were based on CT1 Diet the constituents and supplier are detailed in Appendix B. The experimental diets were prepared in 30kg batches using 1, 2 or 4 premixes.

An appropriate quantity of test compound was added to 1kg of CT1 diet and mixed in a pestle and mortar before being made up into a premix.

The amount of DEHA to be added to the 30kg diet to obtain the required dietary concentration (making allowance for the purity (99.2%) of the DEHA was as follows:

Group	Dose Level of DEHA (ppm)	g of DEHA
1	0	0
2	300	9.07
3	1800	54.44
4	12000	362.90

Test substance and diet were mixed in a Pharma Matrix Blender Model PMA 150 (T K Fielder Ltd) for a 2 minute period.

The prepared diets were dispensed into glass jars via an automated system supplied by Autopak Ltd and the jars fitted with a stainless steel 'follower' perforated with 14 x 1cm diameter holes and a lid with a central aperature of 10cm diameter. These prevent excessive wastage of diet while allowing ready access for feeding. The jars were then stored in plastic trays, each tray held 16 jars.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX C - continued

PREPARATION OF EXPERIMENTAL DIETS

The trays of jars were then labelled with the compound name, study number and concentration and in addition the trays carried a coloured label and the jars had a coloured lid as follows:

Group	Dose Level (ppm)	Colour Code
1	0	Blue
2	300	Green
3	1800	Yellow
4	12000	Red

Trays were kept in an area designated for diet storage until required for the study.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX D

THE DETERMINATION OF DEHA IN DIET BY VORTEX EXTRACTION

METHOD SUMMARY

Accurately weighed diet samples were extracted with hexane on a vortex mixer. Extract solutions were diluted if required to give solutions containing nominally 144-150µg/ml DEHA.

These solutions were analysed by capillary gas chromatography with a flame-ionisation detector. The areas of the peaks due to DEHA were used to calculate the dietary concentration.

CHEMICALS

Hexane, HPLC grade - Rathburn Chemicals.

CALIBRATION STANDARDS

Preparation of Stock Solution

DEHA (nominally 250mg), CTL reference Y02259/003/001, purity 99.2% w/w, was accurately weighed into a 50ml standard flask, the test substance dissolved in hexane and diluted to 50ml (nominally 5mg/ml).

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX D - continued

THE DETERMINATION OF DEHA IN DIET
BY VORTEX EXTRACTION

Preparation of Working Standard Solutions

Portions of the stock solution (2.0, 3.0, 4.0 and 5.0ml) were each diluted to 100ml with hexane to give solutions containing nominally 100, 150, 200 and 250µg/ml DEHA respectively.

PROCEDURE

(a) Preparation of Recovery Diet Samples

Typically these were prepared as follows:-

300ppm

DEHA (nominally 75mg), CTL reference YO2259/003/001 was dissolved in hexane and diluted to 25ml in a standard flask (3mg/ml). Portions (200µl) of this solution were added to each of three 2g amounts of control diet. After mixing with a glass pasteur pipette the diets were allowed to stand at room temperature overnight.

1800ppm

DEHA (nominally 450mg), CTL reference YO2259/003/001 was dissolved in hexane and diluted to 25ml in a standard flask (18mg/ml). Portions (200µl) were added to triplicate 2g amounts of control diet and treated as described above.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX D - continued

THE DETERMINATION OF DEHA IN DIET
BY VORTEX EXTRACTION

12000ppm

DEHA (nominally 24mg), CTL reference Y02259/003/001 was weighed into glass tubes. Control diet (2g) was added and the tube contents mixed with a glass pasteur pipette.

(b) Extraction

Approximately 10g portions of each test diet were ground using a pestle and mortar. Duplicate 2g portions of the ground sample were accurately weighed into screw-capped glass tubes. To control and 300ppm diet, 4.0ml hexane was added. To diets at other levels, 5ml hexane was added. Samples were vortex mixed (Gallenkamp Spin Mix) for 60 seconds, then centrifuged for 10 min at 1500rpm (MSE Mistral 4L). Extract solutions were transferred to vials and diluted with hexane if required to give solutions containing nominally 144-150µg/ml DEHA.

(c) Gas-liquid Chromatography

Gas Chromatograph	: Carlo Erba HRGC 5300 Mega Series or a Pye Unicam 204.
Column	: 007 series bonded phase fused silica capillary column, 15m x 0.53mm id, 1.0µ film thickness, methyl 50% phenyl silicone (Quadrex Corporation).
Column Oven Temperature	: Typically 210°C, hold for 1 min, programmed at 12°C/min to 240°C, hold for 4 min. Minor variations on these conditions were used on occasions.
Detector	: Flame-ionisation.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX D - continued

THE DETERMINATION OF DEHA IN DIET
BY VORTEX EXTRACTION

Detector Oven Temperature:	300°C
Carrier Gas	: Helium, 1kg/cm^2
Make Up Gas	: Argon/methane, 95:5v/v, 0.7kg/cm^2
Detector Gases	: Hydrogen 0.8kg/cm^2, air 1.5kg/cm^2
Injection	: 1µl, HOT injector (Carlo Erba) on-column cooling for 30 seconds.
Data Handling	: Trilab 2000 (Trivector Scientific).

Alternative conditions employed were as follows:-

Gas Chromatograph	: Pye Unicam 204
Column	: BP1, 15m x 0.53mm id fused silica, 3µm film thickness
Column Temperature:	210°C, hold for 1 min, programmed at 12°C/min to 250°C, hold for 2 min.
Carrier Gas	: Nitrogen, 7 lb/in^2
Injection	: 2µl, manual

Sample solutions and the calibration standard solutions were transferred to vials. These were arranged on the sample carousel so that all the calibration standard solutions were injected at the start of a run and the 150µg/ml standard interspersed at regular intervals during the run. The mean peak area value was calculated for each sample solution. A calibration graph was constructed by input of mean peak area values for standard solutions to a statistics computer programme to produce a linear regression plot. The mean peak area values obtained for sample extract solutions were then entered and a concentration value (Cµg/ml) obtained. Alternatively concentrations were calculated by direct proportion to a bracketed mean peak area value obtained for the 150µg/ml standard.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX D - continued

THE DETERMINATION OF DEHA IN DIET
BY VORTEX EXTRACTION

CALCULATION OF RESULTS

(a) Recovery Determination

The following equation was used to calculate the % recovery of DEHA in diet:-

$$\% \text{ recovery} = \frac{C_S \times D_F \times 100}{W \times T}$$

C_S = concentration of DEHA in analysed recovery samples (µg/ml)
D_F = dilution factor (ml)
W = sample weight (2g)
T = concentration of recovery samples (ppm w/w)

(b) Calculation of the Dietary Levels of DEHA

The following equation was used to calculate the level of incorporation of DEHA in diet:-

$$\text{ppm (w/w) DEHA} = \frac{C_S \times D_F \times P}{2 \times R}$$

C_S = concentration of DEHA in analysed samples (µg/ml)
D_F = dilution factor (ml)
P = purity of reference material (99.2% w/w)
R = % recovery

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX D - continued

THE DETERMINATION OF DEHA IN DIET BY VORTEX EXTRACTION

LIMIT OF DETERMINATION

The limit of determination was set at 10ppm for DEHA in diet.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX E

THE DETERMINATION OF DEHA IN DIET BY SOXHLET EXTRACTION

METHOD SUMMARY

Accurately weighed diet samples were Soxhlet extracted with hexane. The extract solutions were diluted with hexane to give solutions containing nominally 108-120µg/ml DEHA.

These solutions were analysed by capillary gas chromatography with a flame-ionisation detector. The areas of the peaks due to DEHA were used to calculate dietary concentrations.

CHEMICALS

Hexane, HPLC grade - Rathburn Chemicals.

CALIBRATION STANDARDS

Preparation of Stock Solution

DEHA (nominally 150mg), CTL reference YO2259/003/001, purity 99.2% w/w was accurately weighed into a 50ml standard flask, dissolved in hexane and diluted to 50ml (nominally 3mg/ml).

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX E - continued

THE DETERMINATION OF DEHA IN DIET
BY SOXHLET EXTRACTION

Preparation of Working Standard Solutions

Portions of the stock solution (1.0, 2.0, 3.0 and 4.0ml) were each diluted to 50ml with hexane to give solutions containing nominally 60, 120, 180 and 240µg/ml DEHA respectively.

PROCEDURE

(a) Preparation of Recovery Diet Samples

300ppm

Aliquots (1.0ml) of the DEHA stock solution were added by pipette to each of three 10g portions of control diet contained in 100ml beakers. The diets were stirred with glass rods, left for at least 2 hours, then transferred with a small volume of hexane to Soxhlet extraction thimbles (22 x 80mm).

1800ppm

Accurately weighed portions (nominally 18mg) of DEHA (YO2259/003/001) were weighed into three 100ml beakers. Control diet (10g) was added, the contents stirred with a glass rod and transferred to extraction thimbles with a small volume of hexane.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX E - continued

THE DETERMINATION OF DEHA IN DIET
BY SOXHLET EXTRACTION

12000ppm

An accurately weighed portion (nominally 1200mg) DEHA (Y02259/003/001) was weighed into a 100ml beaker. A 100g portion of control diet was weighed separately. DEHA was transferred with added portions of control diet to a pestle and mortar to effect a quantitative transfer. The mixture was ground for approximately 5 minutes to obtain a fine intimate mix and finally mixed on a Stuart Flask Rotator for 30 minutes at Speed 6 in a 500ml stoppered conical flask. Three 10g portions were weighed into extraction thimbles.

(b) Extraction

Duplicate 10g portions of diet were weighed into Soxhlet extraction thimbles (22 x 80mm) and these transferred to Soxhlet extractors. Hexane (100ml) was added to 250ml round-bottomed flasks and the necessary components assembled to allow Soxhlet extraction to take place. Samples were extracted for 3 hours and the extract solutions evaporated to approximately 10ml by rotary evaporation under reduced pressure. Extract solutions were transferred with hexane to appropriate standard volumetric flasks and diluted to volume with hexane. Further dilutions in hexane were carried out if required to give solutions containing nominally 120µg/ml (300, 12000ppm) or 108µg/ml DEHA (1800ppm). Control diet extracts were treated in the same manner as the 300ppm samples.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX E - continued

THE DETERMINATION OF DEHA IN DIET
BY SOXHLET EXTRACTION

(c) Gas-liquid Chromatography

Gas Chromatograph	: Carlo Erba HRGC 5300 Mega Series.
Column	: 007 series bonded phase fused silica capillary column, 15m x 0.53mm id, 1.0μ film thickness, methyl 50% phenyl silicone (Quadrex Corporation).
Column Oven Temperature	: 210°C, programmed to 240°C at 12°C/min, hold for 4 min. Alternatively, 200°C hold for 1 min, programme at 10°C/min to 240°C hold for 3 mins. Minor variations on these conditions were occasionally used.
Detector	: Flame-ionisation.
Detector Oven Temperature	: 300°C
Carrier Gas	: Helium at 1kg/cm^2
Make Up Gas	: Argon/methane (95:5 v/v) at 0.7kg/cm^2
Detector Gases	: Air (1.5kg/cm^2), hydrogen (0.8kg/cm^2)
Injection	: 1μl, HOT injector (Carlo-Erba), on-column cooling for 30 seconds.
Data Handling	: Trilab 2000 (Trivector Scientific).

Alternative conditions employed were as follows:-

Gas Chromatograph	: Pye Unicam 204
Column	: BP1, 15m x 0.53mm id fused silica, 3μm film thickness
Column Temperature	: 210°C, hold for 1 min, programmed at 12°C/min to 250°C, hold for 2 min.
Carrier Gas	: Nitrogen, 7 lb/in^2
Injection	: 2μl, manual

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX E - continued

THE DETERMINATION OF DEHA IN DIET
BY SOXHLET EXTRACTION

Sample solutions and the calibration standard solutions were transferred to vials. These were arranged on the sample carousel so that all the calibration standard solutions were injected at the start of a run and the 120µg/ml standard interspersed at regular intervals during the run. The mean peak area value was calculated for each sample solution. A calibration graph was constructed by input of mean peak area values for standard solutions to a statistics computer programme to produce a linear regression plot. The mean peak area values obtained for sample extract solutions were then entered and a concentration value (Cµg/ml) obtained. Alternatively concentrations were calculated by direct proportion to a mean peak area value obtained for the 120µg/ml standard.

CALCULATION OF RESULTS

(a) Recovery Determination

The following equation was used to calculate the % recovery of DEHA in diet:-

$$\% \text{ recovery} = \frac{C_S \times D_F \times 100}{W \times T}$$

C_S = concentration of DEHA in analysed recovery samples (µg/ml)
D_F = dilution factor (ml)
W = sample weight (10g)
T = concentration of recovery samples (ppm w/w)

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX E - continued

THE DETERMINATION OF DEHA IN DIET
BY SOXHLET EXTRACTION

(b) Calculation of the Dietary Levels of DEHA

The following equation was used to calculate the level of incorporation of DEHA in diet:-

$$\text{ppm (w/w) DEHA} = \frac{C_S \times D_F \times P}{10 \times R}$$

C_S = concentration of DEHA in analysed samples (µg/ml)
D_F = dilution factor (ml)
P = purity of reference material (99.2% w/w)
R = % recovery

LIMIT OF DETERMINATION

The limit of determination was set at 10ppm for DEHA in diet.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX F

ARRANGEMENT OF ANIMALS AND GROUPS ON THE RACKS

	RACK 1				RACK 2			
Replicate	Female	Male	Female	Male	Female	Male	Female	Male
1	151-152 (4)	136 (4)	61-62 (2)	46 (2)	106-107 (3)	91 (3)	16-17 (1)	1 (1)
2	108-109 (3)	92 (3)	18-19 (1)	2 (1)	153-154 (4)	137 (4)	63-64 (2)	47 (2)
3	20-21 (1)	3 (1)	110-111 (3)	93 (3)	65-66 (2)	48 (2)	155-156 (4)	138 (4)
4	67-68 (2)	49 (2)	157-158 (4)	139 (4)	22-23 (1)	4 (1)	112-113 (3)	94 (3)
5	159-160 (4)	140 (4)	114-115 (3)	95 (3)	24-25 (1)	5 (1)	69-70 (2)	50 (2)

	RACK 3				RACK 4			
Replicate	Female	Male	Female	Male	Female	Male	Female	Male
6	26-27 (1)	6 (1)	71-72 (2)	51 (2)	116-117 (3)	96 (3)	161-162 (4)	141 (4)
7	118-119 (3)	97 (3)	163-164 (4)	142 (4)	73-74 (2)	52 (2)	28-29 (1)	7 (1)
8	75-76 (2)	53 (2)	30-31 (1)	8 (1)	165-166 (4)	143 (4)	120-121 (3)	98 (3)
9	122-123 (3)	99 (3)	32-33 (1)	9 (1)	167-168 (4)	144 (4)	77-78 (2)	54 (2)
10	34-35 (1)	10 (1)	79-80 (2)	55 (2)	124-125 (3)	100 (3)	169-170 (4)	145 (4)

Group numbers are given in parentheses.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX F - continued

ARRANGEMENT OF ANIMALS AND GROUPS ON THE RACKS

	RACK 5				RACK 6			
Replicate	Female	Male	Female	Male	Female	Male	Female	Male
11	171-172 (4)	146 (4)	126-127 (3)	101 (3)	81-82 (2)	56 (2)	36-37 (1)	11 (1)
12	83-84 (2)	57 (2)	173-174 (4)	147 (4)	38-39 (1)	12 (1)	128-129 (3)	102 (3)
13	40-41 (1)	13 (1)	175-176 (4)	148 (4)	85-86 (2)	58 (2)	130-131 (3)	103 (3)
14	132-133 (3)	104 (3)	87-88 (2)	59 (2)	177-178 (4)	149 (4)	42-43 (1)	14 (1)
15	179-180 (4)	150 (4)	44-45 (1)	15 (1)	134-135 (3)	105 (3)	89-90 (2)	60 (2)

Group numbers are given in parentheses.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX G

ALLOCATION OF FIRST GENERATION (F_0) PARENT RATS
TO EXPERIMENTAL GROUPS

The rats were distributed amongst the four experimental groups, after ensuring that any litters containing unhealthy individuals and litters at the extremes of the weight range were excluded from the randomisation procedure. Cards were numbered 1 to x where x was the number of litters with at least four rats in the litter. The cards were shuffled and a card placed on the cage of each litter to give the order of allocation of the litters to the replicates.

Allocation from within litters was also at random. This was done by numbering cards to correspond with the number of cages in a replicate. Blank cards were also included so that the total number of cards equalled the number of rats in a litter. A card indicating the cage was picked out of a "hat" and simultaneously a rat was picked out of the first litter of females at random. The rat was then allocated to the appropriate cage in the first replicate after it had been ear punched, with its experimental number. Those pups drawn against a blank card were not used. This procedure was carried out until all the female cages in all replicates contained one female rat and then repeated to allocate the second female to each cage. The same procedure was used to allocate one male to the remaining cages in each replicate. Records were kept in the study diary of parentage, date of birth and source of each rat.

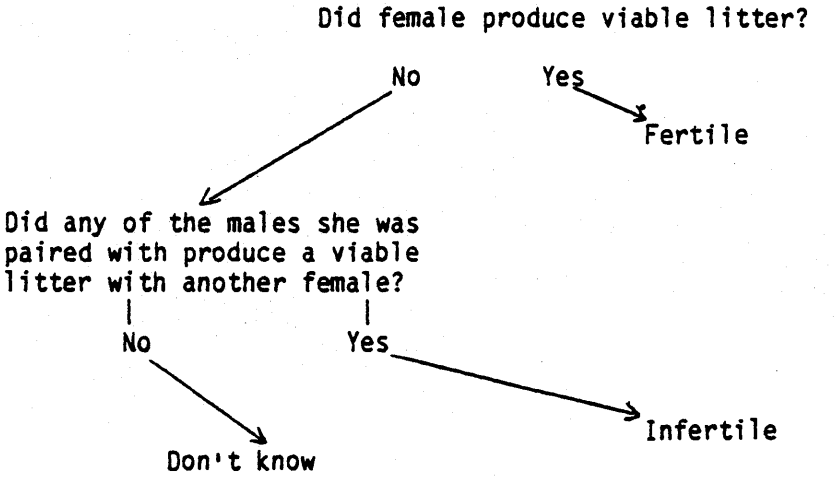
The excess rats were discarded.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX H

THE DETERMINATION OF FERTILITY

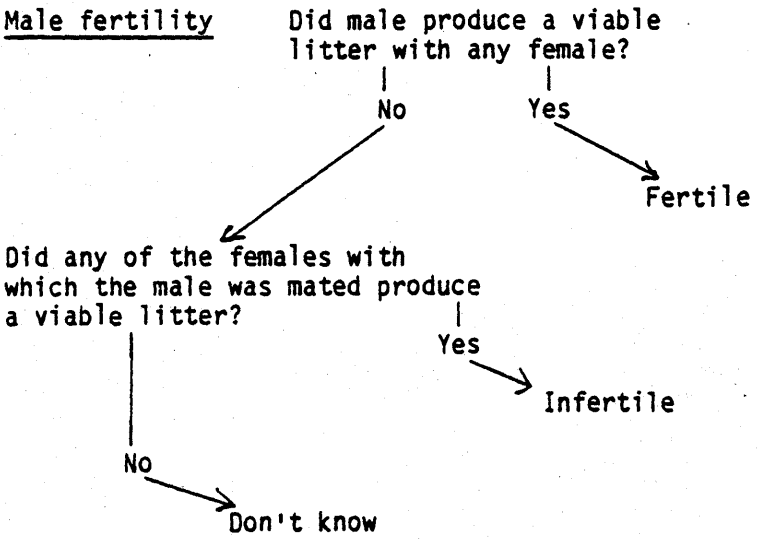
Female Fertility



The don't knows are ignored.

$$\text{female fertility index} = \frac{\text{No fertile} \times 100\%}{\text{No fertile} + \text{infertile}}$$

Male fertility



The don't knows are ignored.

$$\text{Male fertility index} = \frac{\text{No fertile} \times 100\%}{\text{No fertile} + \text{infertile}}$$

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX I

STATISTICAL ANALYSES

Parental bodyweight gain from the start of the study to each week, final bodyweight, weekly food consumption, total food consumption and food utilisation during the premating period were considered by analysis of variance, separately for males and females.

Initial bodyweight, final bodyweight and bodyweight gain of females during pregnancy were considered by analysis of covariance, the covariance representing the effect of re-mating (ie re-mated females tend to have higher bodyweights during pregnancy).

Parental organ weights were considered by analysis of variance and analysis of covariance on last measured bodyweight, separately for males and females.

The proportion of fertile animals in each treated group was compared with the control group proportion using Fisher's Exact Test, separately for each sex. Where it was not possible to establish whether individual animals were fertile or infertile, these animals were excluded from consideration in the fertility indices.

The proportion of viable litters with all pups lost by Day 36 (whole litter losses) in each treated group was compared with the control group proportion using Fisher's Exact Test.

The proportion of females with gestation length (measured in days from date of positive smear to date of birth) of <22, 22 and >22 days and the proportion of matings (for all matings producing a positive smear) with pre-coital interval of length 1, 2, 3, 4 and >4 days were considered by comparing each treated group proportion with the control group proportion using Fisher's Exact Test.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX I - continued

STATISTICAL ANALYSES

Viable litter size and total litter weight on days 1, 5, 11, 22, 29 and 36, mean pup weight on days 1 and 36 and mean pup weight gain to days 5, 11, 22, 29 and 36 were considered by analysis of variance. Analysis of mean pup weight gain and initial and final mean pup weight only were carried out separately for male and female pups.

The proportion of litters with all pups alive on Day 1[a] and the proportion of litters with all pups that are alive on Day 1 and surviving to Day 22[b] were considered by comparing each treated group proportion with the control group proportion using Fisher's Exact Test.

The analyses of litter size, mean pup weight gain, total litter weight and pup survival were also analysed omitting whole litter losses.

Analyses of variance and covariance allowed for the replicate structure of the study design. Except for food consumption and food utilisation analyses allowed also for litter of origin. All analyses of variance and covariance were carried out using the GLM procedure in SAS (1985) and unbiased estimates of treatment group means were provided by the least square means (LSMEANS option in SAS). Each treatment group mean was compared with the control group mean using Student's t-test, based on the error mean square in the analysis. All the analyses were two-sided, except for fertility, live born analysis and survival analysis which were one-sided.

a. Calculated for each litter as:-

$$\text{Proportion of pups live born} = \frac{\text{number of pups born live}}{\text{number of pups born live + number of pups born dead}} \times 100\%$$

b. Calcuated for each litter as:-

$$\text{Proportion of pups surviving to Day 22} = \frac{\text{number of live pups on Day 22}}{\text{number of live pups on Day 1}} \times 100\%$$

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX J

ACHIEVED CONCENTRATIONS IN DIET

Preparation Date	Nominal Concn (ppm w/w)	Analysed Concn (ppm w/w)	Mean Analysed Concn (ppm w/w)	% of Nominal
5 Aug 87†	0 (Control)	ND		
	300	348,315	332	110.7
	1800	1845,1809	1827	101.5
	12000	12450,12770	12610	105.1
23 Aug 87	0 (Control)	ND		
	300	289,292	291	97.0
	1800	1787,1816	1802	100.1
	12000	11650,12300	11980	99.8
12 Sep 87†	0 (Control)	ND		
	300	339,333	336	112.0
	1800	1801,1754	1778	98.8
	12000	10910,10920	10920	91.0
30 Sep 87†	0 (Control)	ND		
	300	300,294	297	99.0
	1800	1904,1791	1848	102.7
	12000	11270,10860	11070	92.3
7 Oct 87	0 (Control)	ND		
	300	289,294	292	97.3
	1800	1782,1671	1727	95.9
	12000	11880,11780	11830	98.6

ND = not detected, detection limit 10ppm.

†These analyses were carried out using a rapid vortex extraction on 2g samples.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX J - continued

ACHIEVED CONCENTRATIONS IN DIET

Preparation Date	Nominal Concn (ppm w/w)	Analysed Concn (ppm w/w)	Mean Analysed Concn (ppm w/w)	% of Nominal
31 Oct 87	0 (Control)	ND		
	300	286,288	287	95.7
	1800	1895,1845	1870	103.9
	12000	11860,12330	12100	100.8
3 Dec 87	0 (Control)	ND		
	300	294,297	296	98.7
	1800	1810,1859	1835	101.9
	12000	12380,12380	12380	103.2
7 Jan 88	1800	1930,1977	1954	108.6

ND = not detected, detection limit 10ppm.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX K

DOSING FORMULATION CHEMICAL STABILITY

Preparation Date	Nominal Concn (ppm w/w)	Extraction Date	Analysis Interval (days)	Analysed Concn (ppm w/w)	Mean Concn (ppm w/w)	% Initial Value
5 Aug 87	300	6 Aug 87†	0	348,315	332	100.0
		24 Aug 87	18	271,329	300	90.4
		2 Sep 87	27	325,292	309	93.1
	12000	6 Aug 87†	0	12450,12770	12610	100.0
		24 Aug 87	18	12080,11720	11900	94.4
		2 Sep 87	27	11990,11620	11810	93.7
23 Aug 87	300	24 Aug 87	0	289,292	291	100.0
		2 Sep 87	9	309,262	286	98.3
		9 Sep 87	16	226,285	256	88.0
		23 Sep 87	30	277,255	266	91.4
	12000	24 Aug 87	0	11650,12300	11980	100.0
		2 Sep 87	9	12180,12160	12170	101.6
		9 Sep 87	16	12090,11740	11920	99.5
		23 Sep 87	30	11710,11310	11510	96.1

† These analyses were carried out using a rapid vortex extraction on 2g samples. Subsequent work showed that whilst this technique appears satisfactory with freshly prepared diet, low results were obtained on aged diet. Therefore all subsequent analysis of samples for stability was performed using the Soxhlet extraction method.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX K - continued

DOSING FORMULATION CHEMICAL STABILITY

Preparation Date	Nominal Concn (ppm w/w)	Extraction Date	Analysis Interval (days)	Analysed Concn (ppm w/w)	Mean Concn (ppm w/w)	% Initial Value
31 Oct 87	300	3 Nov 87	0	286,288	287	100.0
		19 Nov 87	16	285,293	289	100.7
		7 Dec 87	34	290,285	288	100.3
	12000	3 Nov 87	0	11860,12330	12100	100.0
		19 Nov 87	16	11680,12070	11880	98.2
		7 Dec 87	34	11840,12180	12010	99.3

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX L

HOMOGENEITY IN DIET

Nominal Concn (ppm w/w)	Sampling Point	Analysed Concn (ppm w/w)	Mean Analysed Concn (ppm w/w)	Overall Mean Concn (ppm w/w)	% Deviation
300†	Tray 1	282,284	283	285	-0.7
	Tray 3	277,272	275		-3.5
	Tray 5	290,301	296		+3.9
12000†	Tray 1	11930,11850	11890	11510	+3.3
	Tray 3	11530,11550	11540		+0.3
	Tray 5	11380,10820	11100		-3.6

† Values determined by the vortex extraction method.

% Deviation = deviation of mean concentration from the overall mean concentration.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX M

DOSE RECEIVED

The dose of DEHA received was calculated from the group mean bodyweight and group mean food consumption for the relevant week using the following formula:

$$\text{Nominal dietary concentration of DEHA (ppm)} \times \frac{\text{group mean food consumption for week}}{\text{group mean bodyweight for week (g)}} \text{ (g/rat/day)}$$

Since the rats were weighed weekly, the group mean bodyweight was calculated as:

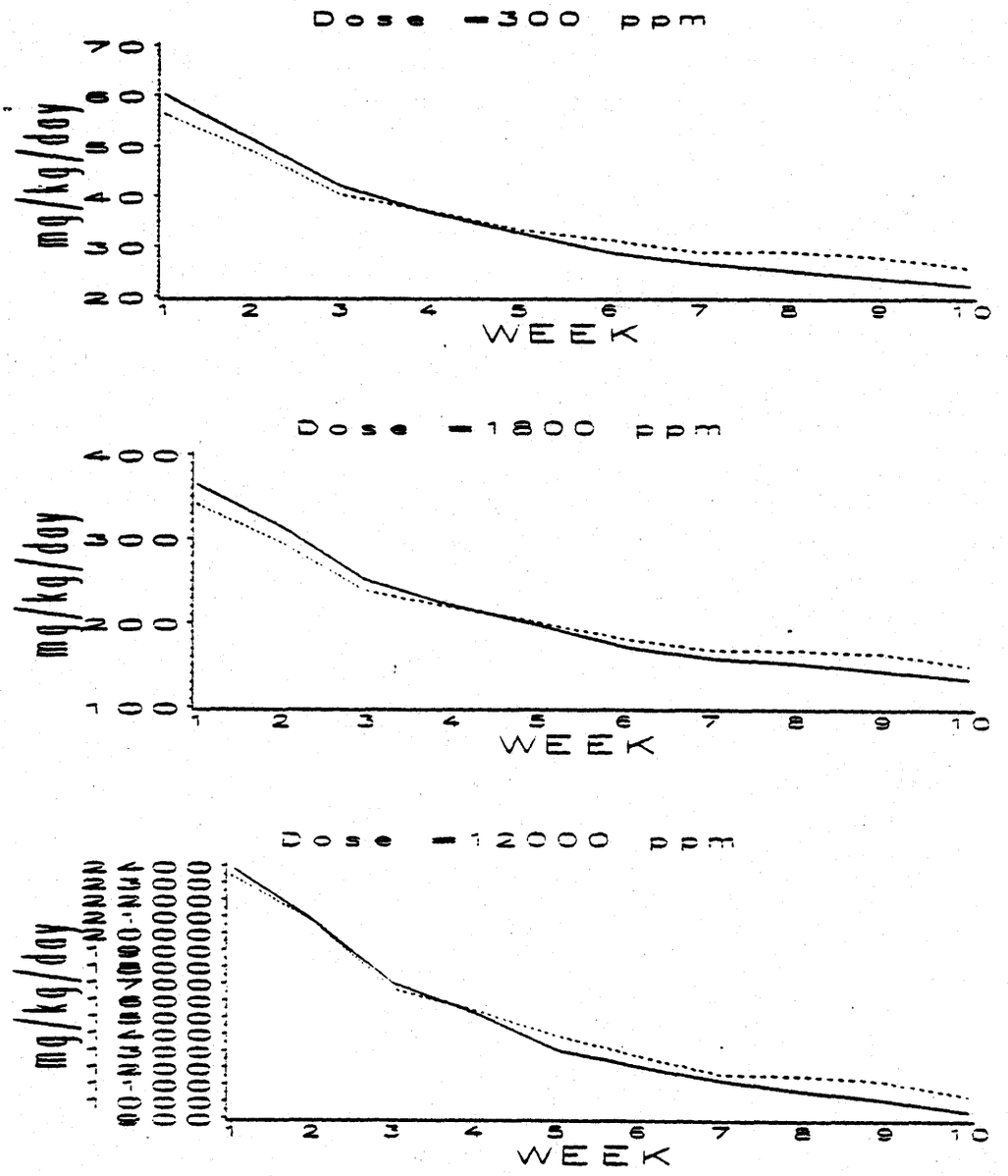
$$\frac{\text{group mean bodyweight at beginning of week} + \text{group mean bodyweight at end of week}}{2}$$

No correction was made for any variation from nominal detected analytically.

DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

APPENDIX M - continued

DOSE RECEIVED PLOTS - F_0 PARENTS



DI-(2-ETHYLHEXYL) ADIPATE (DEHA): FERTILITY STUDY IN RATS

CIRCULATION

Internal

1 Report Centre Reference Copy
2 Report Centre - Spare
3 Dr I F H Purchase)
Dr S E Jaggers)
Dr R S Morrod)
4 Dr G T Steel
5 Dr G H Piggot/Dr M D Stonard/Dr G A Wickramaratne
6 Mr D J Tinston
7 Mrs S Moreland
8 Mr P B Banham
9 Mr S Cook

External

10 Dr B Berndtsson, Neste OXO AB, Sweden
11 Dr F Carpanini, BP International Ltd, England
12 Dr J Jackson, Monsanto Europe, Belgium
13 Dr R Jackh, BASF Toxicology, FRG
14 Dr R J Millischer, Atochem, France
15 Dr J Rudolph, Huls, FRG
16 Dr C Cella, EVC, Belgium
17 Mr N Sarginson, Exxon Chemicals, Belgium
18 Dr D F Cadogan, ICI C&P Group, England
19 Dr C Schneider, BASF, FRG
20 Dr W Pump, Bayer Ag, FRG
21 Dr M Wooder, Shell International, Belgium
22 Dr D Starck, Hoechst Ag, FRG
23 Dr D M Pugh, BP Chemicals, England
24 Mr C R Perry, Monsanto, Belgium
25 Dr A Seys, CEFIC, Belgium

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